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Studies on the myxobacteria: I, Distribution in Iowa soils and description of a new species; II, Myxobacteria as bacterial parasites; III, The morphology and cytology of Myxococcus xanthus sp.n.

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NOTE TO USERS

STUDIES ON THE MYXOBACTERIA.

I. DISTRIBUTION IN IOWA SOILS AND DESCRIPTION OF A NEW SPECIES.

II. MYXOBACTERIA AS BACTERIAL PARASITES.

III. THE MORPHOLOGY AND CYTOLOGY OF *MYXOCOCCUS XANTHUS* SP.N.

by

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I. DISTRIBUTION IN IOWA SOILS AND DESCRIPTION OF A NEW SPECIES.[1]

With the development of their technique for the isolation of myxobacteria the Krzemieniewskis (1926) found that many species previously thought to be more or less obligate coprophiles could be cultivated from the soil. In much of their work they relied solely on soil samples and were able not only to re-isolate many species that had been found on dung, but to discover several new species. In undertaking the present work a modification of the Krzemieniewski technique was employed.

METHODS OF ISOLATION

Samples of soil, and of dung of various kinds, from several localities, were collected in small large-mouthed bottles fitted with screw caps. The soil samples were taken from the upper four or five centimeters since experience had shown that very few myxobacteria were to be found below the top layer of the ground. Dung that had lain on the ground for some time was found to harbor larger numbers of myxobacteria than fresher material. The soil, or dung,

[1] Iowa State College Journal of Science, April 1941.

after being collected, was broken up into small particles. Sieving of the soil was often resorted to in order to eliminate stones, grass and other undesirable materials.

Petri dishes were prepared by placing two or three pieces of filter paper in the bottoms, to aid in retaining the moisture, and enough of the sample added to approximately half fill the plate. Twenty-five to forty pieces of rabbit dung were then placed on top of the soil, or dung, and water added up to 75 to 90 per cent saturation. Various incubation temperatures were tried, but the most satisfactory was found to be room temperature, 20° - 25° C. When held there the development of fruiting bodies on the dung was more normal, though possibly a little slower, than at a somewhat higher temperature.

During the first three to four days molds developed on the plates, in some cases in such large numbers as to make the plates of no further use. However, in most cases the molds reached maximum growth within five days, after which fruiting bodies of some species of myxobacteria began to develop. Plates not completely overgrown by molds were examined daily under a low-power binocular microscope, and when myxobacterial fruiting bodies were found they were transferred to plates of dung decoction agar that had previously been prepared.

This medium was made by adding to 100 grams of fresh rabbit dung one liter of distilled water. The mixture was heated to boiling, and then allowed to infuse for 24 hours. It was then filtered through several layers of cheese cloth, the filtrate made up to volume, and 1.5 per cent Bacto agar added. Sterilization was effected by autoclaving at 15 pounds pressure for 30 minutes. When cooled to about 60° C. plates were poured.

The primary transfers, of which 127 were made from the various soils and dungs, were, of course, not pure cultures. Often molds were carried along on the needle, and these frequently outgrew the myxobacteria. Associated bacteria of various types were also present and usually developed first. These, too, occasionally grew rapidly enough to completely discourage the growth of the more slowly growing slime bacteria. However, fruiting bodies were sometimes able to develop, and were first seen on the tops of colonies of the associated types. Attempts to transfer parts of these fruiting bodies to new media usually resulted in transferring both the myxobacteria and the associate. The appearance of some of these primary transfers after 7 to 10 days incubation is shown in Figures 7, 8, and 10. The fruiting bodies are seen to be developing on the surface of colonies of associated types. By allowing a longer period of incubation pure cultures could be obtained from the thin, nearly transparent

vegetative slime colony that developed beyond the margins of the colonies of associated bacteria. Up to two weeks incubation was often required for the development of the vegetative colonies of myxobacteria, and the fruiting bodies that eventually formed on it were usually smaller and less perfectly formed than those produced directly on the host colony. This fact may be noted in Figure 7. These observations led to the development of a somewhat improved method for isolation.

After molds had been eliminated fruiting bodies were transferred to a cell suspension medium. This medium was prepared by growing large numbers of true bacteria (Escherichia coli, Bacillus subtilis, Serratia marcescens, etc.) in flasks of nutrient broth. After a good growth had been obtained the suspensions were shaken well, in order to distribute the cells evenly throughout the suspension, and 1.5 per cent agar added. The flasks were then autoclaved at 15 pounds pressure for 30 minutes. Transfers were made to plates of this medium. The growth of the myxobacteria was greatly enhanced, and the colonies of vegetative cells developed much more rapidly than did those of associated types. A later modification of this method consisted of growing large numbers of true bacteria on agar, scraping the growth from the agar and washing free of any adhering nutrient materials. An angle-head centrifuge was used for the purpose. After three washings in distilled water the

cells were concentrated, the supernatant liquid poured off, and the paste dried *in vacuo*. The dried cells were then suspended in 1.5 per cent plain agar (no other nutrient materials were added), and the medium sterilized as usual. This medium produced vigorous growth of the myxobacteria, while the associated species failed, in nearly every case, to develop at all. This made the isolation procedures a matter of a few days, rather than two or three weeks.

When pure cultures had finally been obtained they were transferred to dung plates. These were prepared by placing two or three pieces of rabbit dung in each petri dish and sterilizing for one hour at 15 pounds pressure in the autoclave. A 1.5 per cent solution of Bacto agar in distilled water was added to each plate to a depth equal to about one-half the diameter of the pellets of dung. The dung was then moved to the center of the plates with a sterile needle or forceps before the agar solidified. Care was taken not to cover the dung, but to keep the top side of each piece free from agar. It was found that much better growth could be had directly on the dung than on agar-incrusted dung. Transfers were made to the dung, the agar acting to hold it in place and to preserve the moisture content. Growth of all species was found to be good on this medium, and the fruiting bodies produced were large and appeared more nearly normal than on most other media that had been tried. This medium

has been used successfully for carrying stock cultures over periods of many months. Transfers are found to be necessary about once every six to eight weeks, depending upon humidity and temperature.

SPECIES ISOLATED

Undoubtedly conditions vary sufficiently from one country to another to affect, to a high degree, the flora of the soils. It has been the experience of the present author that the majority of types to be found in the soils of the environs of Ames, Iowa, belong to the genus Myxococcus and that other types, while present, do not abound. Private communications with two other persons interested in the group tend to indicate that this is also true in other parts of the United States. Many of the species used for this work were originally found on dung.

Of the 127 primary transfers made, only a small percentage were finally isolated in pure condition. This was chiefly due to the fact that the associated organisms (both bacteria and molds) were able to outgrow the myxobacteria after transfer from the natural substrate. It is of interest to note the variety of types that were observed, with some indication of the natural substrate. A condensed list is given here, with brief descriptions of the species that were isolated in pure culture. These are indicated by the culture numbers

following the names.

Myxococcus fulvus (Cohn emend. Schroeter) Jahn 1, 44, 76, 106. Produces bright pink fruiting bodies, spherical to subspherical, often constricted at the base and supported on a mound or foot of slime; the surface smooth with no outer wall or limiting membrane; up to 350 microns in diameter. Spores spherical, average 1.3 microns diameter.

From wet sand, pasture soil, cow, sheep and goat dung.

Myxococcus fulvus var. albus Jahn 108. Similar to M. fulvus but color much lighter, often giving rise to very pale pink or nearly white fruiting bodies.

From sandy soil.

Myxococcus virescens Thaxter 57, 61, 91, 100. Fruiting bodies somewhat smaller and less regularly spherical than those of M. fulvus. Color yellow to greenish yellow. Spores 2.0 microns diameter.

From wet sand, pasture soil, goat dung.

Myxococcus xanthus[1] Beebe 115, 127. Similar to M. virescens in size and shape of fruiting bodies and spores. Color bright orange.

From pasture soil and cow dung.

Polyangium fuscum (Schroeter) Thaxter 77. Fruiting bodies composed of masses of oval to spherical cysts, each surrounded by a tough, reddish-brown wall; up to 90 x 125 microns. Mass of cysts held together by a colorless, transparent slime envelope. Resting cells, or spores, rod-shaped; about 0.8 x 3.0 microns.

From sand, sandy loam, pasture soil, cow, sheep and rabbit dung.

Podangium erectum (Schroeter) Jahn 82, 104. Reddish-brown cysts, oval or club-shaped, rising from a sort of hypothallus. Cysts single, each with a thick outer wall or membrane; 40 to 50 microns in diameter, up to 100 microns in height. Often 100 or more on one hypothallus. Spores shortened rods, 0.7 x 3.0 microns.

From cow dung and soil from woods and pasture.

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[1]Diagnosis of M. xanthus included in paper submitted to Journal of Bacteriology; in publication at the time of this writing.

CHONDROCOCCUS BLASTICUS sp. n. 90, 111. This organism was at first considered a large variety of M. fulvus due to the marked similarity of the primary fruiting body of this species, both in color and formation, and those of M. fulvus. The formation of secondary fruiting bodies, however, made it quite obvious that the species were distinct.

Etymology: Greek adj. = budding, additional growth.

Diagnosis:

Fruiting body. Primary: Spherical to subspherical, usually sessile but occasionally with a short stalk or foot, pale to bright pink; 300 to 600 microns in diameter. No outer wall or limiting membrane evident. Develops on sterilized rabbit dung in from 3 to 5 days at room temperature. Secondary: Arising as a bud-like growth from the primary fruiting body. Develops into irregularly shaped, finger-, coral- or bud-like protuberances. Seldom branched, occasionally stalked but usually sessile on primary fruiting body. Deep pink to salmon pink in color. Quite variable in size and shape: 50 to 150 x 175 to 425 microns. No outer wall or limiting membrane evident.

Spores. Spherical, thick-walled, highly refractile; 1.2 to 1.4 microns in diameter. Held together in the fruiting body by the mass of slime.

Vegetative colony. Thin, colorless, transparent at margin; surface broken by many small ridges, or veins. Center smoother, slightly thicker, often showing pale pink pigmentation.

Vegetative cells. Long, slender, flexible rods, straight or curved to bent, ends rounded to tapered, gram-negative. Often show one or two deep-staining bodies within, at or near center, while ends of cell stain lightly. 0.5 to 0.6 x 3.0 to 5.0 microns. Usually found in groups of from 2 to 12, lying parallel, the group moving as a unit. Motile by a crawling or creeping motion; no flagella.

Habitat. Observed once on goat dung, once in soil, Ames, Iowa.

SPECIES IDENTIFIED BUT NOT ISOLATED

Chondromyces crocatus Berkeley and Curtis from cow dung.

Chondrococcus coralloides (Thaxter) Jahn from pasture soil and cow dung.

Chondrococcus sp. (yellow) from sheep dung.

Myxococcus sp. (deep red-orange) from sheep dung.

DISCUSSION

Myxococcus fulvus and M. virescens were found to be by far the most numerous species, the fruiting bodies appearing in large numbers on plates of nearly every sample brought into the laboratory. Polyangium fuscum was noted on a wide variety of soils and dungs, but always in small numbers, as was the case with M. xanthus and Podangium erectum. Chondromyces crocatus appeared in moderately large numbers on only one sample of cow dung. Attempts to purify this species failed completely, as was the case with Chondrococcus coralloides and undetermined species of Chondrococcus and Myxococcus. Myxococcus fulvus var. albus was found only once in sandy soil.

Several authors have reported various species of myxobacteria from such materials as bark of trees, dead leaves, straw, etc. These materials were all sampled during the course of the work but no slime bacteria were isolated from any of them.

SUMMARY

An improved method for the isolation of myxobacteria from soils and dungs is described.

A new species, Chondrococcus blasticus, is diagnosed.

The distribution of species of the Myxobacteriales in Iowa soils in the vicinity of Ames is discussed and shown to be fairly general as regards the families Myxococcaceae and Polyangiaceae. By far the most common species were those belonging to the genus Myxococcus, particularly M. fulvus and M. virescens. M. xanthus also appeared on a wide variety of soils and dungs, but in smaller numbers. This was also the case with Polyangium fuscum and Podangium erectum. No species of the families Sorangiaceae or Archangiaceae were observed, though it is not suggested that they are entirely absent from the soils of this region.

II. MYXOBACTERIA AS BACTERIAL PARASITES.[1]

Few of the investigators who have concerned themselves with the myxobacteria have studied the nutritional requirements of the members of this group. The earlier workers observed that most species developed well on dung of one kind or another. Quehl (1906) recorded slow growth of some species which he cultivated on malt extract gelatine, the gelatine becoming liquefied. Good growth occurred on potato nutrient agar, but sterilized potato was not a satisfactory substrate. Kofler (1913) described good growth on a sucrose-peptone agar containing small amounts of magnesium sulphate and potassium phosphate. He regarded peptone as necessary for normal development.

Pinoy (1913) made first mention of a possible symbiotic or parasitic relationship of certain myxobacteria with species of the Eubacteriales. He noted that Chondromyces crocatus failed to develop on any medium unless some organism such as Micrococcus sp. was also present. He concluded the presence of the latter was necessary for the growth of the former.

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[1] Iowa State College Journal of Science, April 1941.

Reporting on the utilization of various carbon compounds by certain species of the Myxobacteriales, Beebe (1940) showed the utilization of both starch and cellulose, the former being broken down by a diffusible extracellular enzyme. He also noted a dependence of the myxobacteria on other bacterial forms: killed suspensions of true bacteria were found to supply all of the nutrient requirements of the slime bacteria. This was suggested as being parasitism rather than symbiosis, and was borne out by the findings of Snieszko, McAllister and Hitchner (1941) who pointed out a lytic action of certain species of myxobacteria on living colonies of gram-negative true bacteria.

The purpose of the present work was to determine, if possible, something of the nature of the nutritional demands, with special emphasis on the relationships with true bacteria, of several species of myxobacteria from soils and dungs of Iowa.

ISOLATION PROCEDURES

The methods used in the isolation of the various species have been previously outlined. In brief they were as follows:

Soil and dung samples, collected in screw-cap jars, were pulverized and sieved, and distributed in petri dishes to a depth of about one-quarter inch. Twenty-five to forty

pieces of rabbit dung were placed on the samples, and water added to about 75 to 90 per cent saturation. The plates were incubated at room temperature (22° - 25° C.) for 10 to 14 days. Molds developed rapidly the first three or four days of incubation, but after their maximum period of growth had passed, fruiting bodies of the myxobacteria began to form on the dung. The plates were examined daily under a low-power binocular microscope. When fruiting bodies were located they were transferred to plates of dung decoction agar. If the fruiting body germinated, and was not overgrown with associated molds and bacteria, an additional transfer to a bacterial cell suspension agar usually completed the isolation procedure. Stock cultures were held on plates of sterilized rabbit dung partially imbedded in plain 1.5 per cent agar.

ASSOCIATED BACTERIA

Forty-six isolations were made of the organisms found associated with the myxobacteria in order to determine whether any particular type played an important part in the growth and development of the slime bacteria. Of the total number only five were spore-forming rods; two were cocci. The remainder were gram-negative, non-spore-forming rods varying in shape from nearly spherical cells less than a

micron long up to rods nearly 4 microns long. In only a very few cases did the pigmentation vary decidedly from white or light yellow; one culture produced pale orange pigment, a few developed a bright yellow color after several days, and two were pale pink or pinkish orange. Except in the case of the cocci, which belonged to the genus Staphylococcus, and in the case of one spore-former, the cells were all single, seldom forming chains or any other definite type of group. On the whole they represented common soil forms; they have not as yet been further identified.

Several of these associated forms were used during the succeeding work. Each has been identified by a number indicating its source, such as Associate No. 100/3: the third associate cultured from primary isolation No. 100.

EXPERIMENTAL PROCEDURES AND RESULTS

Early observations showed what appeared to be a close relationship between the myxobacteria and certain true bacteria. Growth of such organisms as Chondromyces crocatus in mixed culture, especially in the presence of various bacteria and molds, and the ability of this organism to develop only poorly, if at all, under other conditions, checked closely with the work of Pinoy (1913) on the same species. Myxococcus virescens also showed a marked tendency to degenerate when in pure culture, but to revive and to pro-

duce more or less normal fruiting bodies when again transferred to unsterilized rabbit dung. Polyangium fuscum presented a problem in isolation in that it appeared to thrive in what seemed to be an extreme case of association: a very small, motile, gram-negative non-spore-forming rod was noted to live in the slimy outer envelope surrounding the ripened cysts, as well as in the slime of the vegetative colony. Both organisms seemed to benefit by the association and it was only after many months that a separation was achieved. The associate failed to develop on any other medium. P. fuscum was found to grow almost as well without it when inoculated on sterilized rabbit dung, or a bacterial suspension in agar. The development of large, perfectly formed fruiting bodies by most of the species on the tops of colonies of associated species of true bacteria, while those developing beyond the margins of the host colonies were smaller and less perfect, also indicated the importance of the relationship.

However, it was felt that some other factor might have an important role in the nutrition of the slime bacteria; the frequent reference of the earlier workers to growth on dung of all kinds might mean the utilization of fecal types of bacteria by the myxobacteria, the presence of some sort of growth factor in dung (aside from bacteria) particularly favorable for the growth of myxobacteria, or a combination of both.

After several preliminary trials a series of experiments was run in an effort to determine whether the dung itself was necessary to the growth and development of certain species, or whether the factor influencing the myxobacteria was to be found in the bacteria said to compose a large part of dung. Four media were used: 1) nutrient agar containing the usual three grams of beef extract, five grams of peptone and 15 grams of agar per liter; 2) dung decoction agar, made as previously described; 3) bacterial suspension-nutrient agar which was prepared by inoculating one liter of nutrient broth with an actively growing broth culture of associate No. 91/3, a large, non-spore-forming rod. This was incubated at 37.5° C. for 48 hours, during which time it was shaken frequently for purposes of aeration. 1.5 per cent agar was then added and the suspension autoclaved for 30 minutes at 15 pounds pressure. This medium contained both bacterial cells and their metabolic products. 4) Bacterial suspension agar was prepared by growing the same organism, No. 91/3, on large slants (1 x 8 inch tubes) of nutrient agar. After three days incubation at 37.5° C. the heavy growth of twenty slants was removed, suspended in distilled water used for washing the slants clean, and concentrated by centrifugation. Twenty minutes at 3500 R.P.M. in the International Centrifuge was sufficient to throw down nearly all of the cells. They

were then re-washed and centrifuged twice in order to remove any adhering nutrient materials or metabolic products. The washed cells were suspended in 250 cc. of distilled water and plate counts made to compare the concentration of cells with that of the bacterial suspension-nutrient agar medium, plate counts having been previously made on this latter. The cell counts on the washed suspension averaged about twice those made on the nutrient broth suspension. Accordingly, the washed cell suspension was diluted to twice its volume, 500 cc., and 7.5 grams of agar added. Sterilization was in the autoclave at 15 pounds pressure for 30 minutes.

All of these media were used for plate cultures. Inoculations were made by transferring matured fruiting bodies from the stock cultures, rabbit dung plates, by means of a fairly short needle. In order to facilitate the transfer a low-power binocular microscope was used; this made it possible to pick up about the same amount of material on the needle for each transfer, and eliminated, to a large degree, carrying over bits of dung or agar from the stock cultures. The fruiting bodies were implanted on the centers of the plates. Incubation for this first work was at 30° C. for periods of from 6 to 10 days. As a rule 12 to 72 hours were required for the germination of the spores before any growth could be noted; this period depended chiefly on the

species, rather than temperature or medium.

The following species were used in this experiment:

Myxococcus fulvus 44, 76.

M. virescens 57, 61.

Polyangium fuscum 77.

Podangium erectum 82, 104.

Chondrococcus blasticus 90, 111.

The results after seven days are shown in Tables I - IV. Growth was compared, by measuring the diameters of the colonies in millimeters, each day during the incubation period; formation of fruiting bodies is indicated by figures in italics.

Table I

Diameters of Colonies in Millimeters
on Bacterial Suspension Agar
7 days - 30.0° C.

Name and Number	Age in Days 2	3	4	5	6	7
M. fulvus 44	12.5	20.5	28.0	35.0	40.0	46.0
M. virescens 57	6.4	16.0	25.0	30.0	38.0	47.0
M. virescens 61	6.4	13.5	20.0	25.0	28.0	33.0
M. fulvus 76	14.0	22.5	32.0	36.0	42.5	46.0
P. fuscum 77	8.5	8.5	10.0	--	--	--
P. erectum 82	3.5	5.0	10.0	17.0	23.0	30.0
C. blasticus 90	7.2	16.0	23.0	30.0	33.0	38.0
P. erectum 104	3.2	9.5	18.0	30.0	38.0	45.0
C. blasticus 111	12.0	18.0	25.0	30.0	34.0	40.0

Table II

Diameters of Colonies in Millimeters
on Nutrient Agar
7 Days - 30.0° C.

Name and Number	Age in Days 2	3	4	5	6	7
M. fulvus 44	6.5	10.0	15.0	17.5	20.0	22.5
M. virescens 57	5.6	12.0	18.0	23.0	27.0	33.0
M. virescens 61	6.4	10.0	15.0	18.0	21.0	25.0
M. fulvus 76	6.4	9.0	11.5	12.5	13.0	14.0
F. fuscum 77	9.5	10.0	10.0	--	--	--
F. erectum 82	1.5	1.6	5.0	11.0	16.5	24.0
C. blasticus 90	3.6	6.0	8.5	10.0	11.5	14.0
F. erectum 104	--	--	--	--	--	--
C. blasticus 111	4.5	8.0	13.0	14.5	16.0	17.5

Table III

Diameters of Colonies in Millimeters
on Bacterial Suspension-Nutrient Agar
7 Days - 30.0° C.

Name and Number	Age in Days 2	3	4	5	6	7
M. fulvus 44	10.0	13.0	20.0	--	--	--
M. virescens 57	9.0	14.0	21.0	26.0	28.5	33.0
M. virescens 61	9.6	15.0	18.0	25.0	30.0	36.0
M. fulvus 76	8.5	11.5	15.0	16.0	17.5	18.0
F. fuscum 77	--	--	--	--	--	--
F. erectum 82	3.2	3.5	3.5	12.0	19.0	25.0
C. blasticus 90	6.0	13.0	20.0	24.0	26.0	28.0
F. erectum 104	--	--	--	--	--	--
C. blasticus 111	8.5	15.0	21.0	25.0	29.0	32.0

Table IV

Diameters of Colonies in Millimeters
on Dung Decoction Agar
7 Days - 30.0° C.

Name and Number	Age in Days 2	3	4	5	6	7
M. fulvus 44	1.6	2.0	3.0	6.0	7.0	8.0
M. virescens 57	3.6	4.5	8.0	11.0	14.0	21.0
M. virescens 61	5.6	8.0	13.0	17.0	20.0	27.0
M. fulvus 76	5.5	8.0	11.0	15.0	18.0	23.0
P. fuscum 77	6.0	6.2	7.0	--	21.0	31.0
P. erectum 82	3.6	4.5	5.2	7.0	7.5	7.5
C. blasticus 90	--	--	--	--	--	--
P. erectum 104	3.5	3.7	7.0	15.0	20.0	34.0
C. blasticus 111	2.4	2.4	2.4	--	--	--

In order to compare more easily the growth of any one organism on the four media the figures given above have been plotted as growth curves. These are given in Figures 1 to 6. In the case with two strains of Myxococcus fulvus better growth was obtained on the suspension of killed cells alone. In culture No. 44 the second best growth was on the nutrient agar-bacterial suspension combination, followed by plain nutrient agar and lastly by dung decoction agar. With culture No. 76 the three last named media closely approximated each other, all being much less favorable than the bacterial suspension alone. Myxococcus virescens 57 and 61 presented a less striking picture, although in one case there is a significant difference between the colony size on the suspen-

sion of killed cells and that on the nutrient agar, and bacterial suspension-nutrient agar, both of which appeared to produce identical results. Dung decoction agar ranked third in the case of No. 61, fourth in the case of No. 57. Polyangium fuscum 77 failed to grow in three instances, developing only moderately well on dung decoction agar. Podangium erectum 82 and 104 showed a particularly significant difference in rates of growth on bacterial suspension agar and dung decoction agar. Culture No. 82 gave identical results on the two nutrient agars, while 104 failed to develop on either. Chondrococcus blasticus 90 and 111 both grew rapidly, in a nearly straight line curve, on the suspension of killed cells alone, with no apparent decrease in rates of growth during the entire incubation period. On the nutrient agar-cell suspension medium there is seen to be a definite falling off in rate after four days, while the same is true on nutrient agar. The entire latter curve is much lower than either of the preceding. This organism failed to grow on dung decoction agar.

The production of fruiting bodies may also be considered indicative of the value of a particular medium. Seven of the nine strains produced fruiting bodies on cell suspension agar at the end of the period of incubation, while one, M. fulvus 76, had fruited by the third day. Five produced fruiting bodies by the fifth day. After seven days only

three cultures showed fruiting bodies on nutrient agar, and two on each nutrient-cell suspension and dung decoction agars.

A second series of experiments was run to check the first. This time associates No. 82/1 and No. 100/3, a short gram-negative rod and a Staphylococcus respectively, were used in the suspensions. Also, it was thought that the presence of the metabolic products might have some inhibitory effects on the growth of the myxobacteria so that the cells to be suspended were grown on large slants, the growth removed as previously described, and re-suspended in distilled water to which was added 7.5 grams of plain agar in one case, 11.5 grams of prepared Difco Bacto Nutrient agar in the other, to each 500 cc. of suspension. The results, given in condensed form in Table V, after seven days incubation at 30° C., were essentially the same as in the previous trials.

Except for M. virescens 57 and 61, best growth invariably occurred on the suspension of short rods (No. 82/1), and the next most favorable medium in every case was the suspension of Staphylococcus (No. 100/3). A comparison of the two nutrient agars will show little difference with the type of suspended cells. Fruiting body production was higher on the two plain cell suspensions, although there was less difference to be seen here than in the previous trials.

Table V

Diameters of Colonies in Millimeters
on Four Cell Suspension Media
7 Days - 30.0° C.

Name and Number	Associates			
	82/1		100/3	
	Plain	Nutr.	Plain	Nutr.
M. fulvus 44	17	--	15	--
M. virescens 57	27	19	19	25
M. virescens 61	24	27	26	28
M. fulvus 76	22	6	17	12
P. fuscum 77	31	19	23	--
P. erectum 82	42	28	37	24
C. blasticus 90	25	10	22	10
P. erectum 104	37	--	15	9
C. blasticus 111	26	13	21	14

Within 24 hours after the inoculations had been made a clarified area, or window, could, in most cases, be noted around the point of inoculation. As the colonies grew this area was seen to increase in size. It appeared that the cells in the suspensions were being lysed by extracellular enzymes secreted by the myxobacteria. Microscopic examination of agar from within this area showed very few cells remaining in the medium, while the agar outside the growth area revealed the presence of numerous cells intact. In a few cases the lysed area extended several millimeters beyond the margin of the myxobacterial colony, but for the most part the two areas coincided.

Quantitative determinations were then made in an effort to show the effect of varying the concentrations of the cell suspensions. Trial runs, using associate culture No. 100/3 again, indicated a direct relationship between the number of cells in the suspension and the rates of growth of the myxobacteria. In these first experiments the cells for the suspensions were grown on large slants and suspended in distilled water to give concentrations of 80,000,000; 40,000,000; and 20,000,000 cells per cubic centimeter. These were determined by plate counts. No other nutrient materials were added to the suspension in agar. Fruiting body production on the three media was, in general, about the same throughout. This was thought to be due to too little difference between the highest and lowest cell concentrations.

A series of more closely controlled experiments followed. It was felt that better control could be exercised with definite weights of cells rather than approximate numbers of cells, such as result from plate counts. Consequently large numbers of cells were grown in flats--six-ounce Blake bottles to which had been added 20.0 cc. of nutrient agar; these were plugged with cotton, sterilized as usual, and allowed to cool in a horizontal position, giving a large surface of nutrient agar upon which to grow the cells. Inoculations were made by pipette from actively growing 24-hour broth cultures, and the flats incubated at 37.5° C. for two days.

The growth was then scraped off, suspended in distilled water, centrifuged, and then re-washed and centrifuged two additional times. The resulting cell paste was then dried *in vacuo* and stored in a desiccator over calcium chloride.

The following cultures were so treated and used for cell suspensions:

No. 100/4 - Large spore-forming rods, colorless.

No. 89/2 - Small spore-forming rods, colorless.

No. 108B/3 - *Sarcina*, yellow.

No. AB - (a contaminant) - *Serratia*, red.

The purpose of this variety was to determine the effects, if any, of spore-formers and chromogens on the growth of myxobacteria. The cells were ground lightly in an agate mortar to reduce the dried paste to a powder, and added to 1.5 per cent plain agar in the following amounts:

00.0 milligrams per 100.0 cc. agar (control)

25.0 milligrams per 100.0 cc. agar

50.0 milligrams per 100.0 cc. agar

100.0 milligrams per 100.0 cc. agar

The first trial was with suspension 100/4, and the results of this run over a seven day period are shown in Table VI. *Myxococcus fulvus* 76 and 108, *Podangium erectum* 82, *Chondrococcus blasticus* 111 and *Myxococcus xanthus* 127 were the species used for this experiment.

Table VI

Growth on Various Concentrations of a
Spore Forming Bacterium
7 Days at Room Temperature

Mgm. of cells/ 100 cc.	Cult. No.	Age in Days			
		1	3	5	7
00.0	76	1.5	3.7	6.0	7.0
	82	1.0	1.5	2.0	7.0
	108	2.0	2.3	2.5	2.5
	111	1.5	2.0	2.0	2.5
	127	1.5	3.5	5.0	7.0
25.0	76	3.2	8.0	12.0	16.0
	82	2.0	3.0	5.0	6.0
	108	2.0	2.2	5.0	8.0
	111	2.0	7.2	12.0	18.0
	127	---	5.7	10.5	15.5
50.0	76	3.0	9.0	14.0	19.0
	82	1.5	1.5	2.0	12.0
	108	1.5	2.5	6.5	11.0
	111	2.5	8.5	15.0	21.0
	127	2.0	7.0	13.0	19.0
100.0	76	2.5	9.0	14.0	19.0
	82	2.0	3.0	5.0	11.5
	108	2.0	3.5	8.0	14.0
	111	2.0	9.5	16.5	22.0
	127	2.0	9.5	18.0	23.0

Examination of the data reveals a gradual but definite increase in the diameters of the colonies, in nearly every case, proportional to the increase in the cell content of the medium. There is also a corresponding tendency to produce fruiting bodies in the higher cell concentrations, while

none was formed in the lower ones. It also might be noted here that in the cases of M. fulvus 76, P. erectum 82 and M. xanthus 127 growth actually occurred on the plain 1.5 per cent agar which acted as the control. It was thought that the distilled water used in the solutions might possibly contain sufficient minerals to support growth. Additional trials were made using glass-distilled water, but growth on those media closely approximated that shown in the above table.

Table VII gives, in condensed form, the results of growth on the three other cell suspensions previously mentioned. The incubation period also was seven days, and the plates held at room temperature. Myxococcus fulvus 44 was substituted for 76; all other cultures were the same.

Table VII

Summary of Growth on Various Concentrations
of Three Cell Suspensions
7 Days - Room Temperature

Cell Susp. No.	Cult. No.	Mgm. Dried Cells per 100 cc. 00.0	25.0	50.0	100.0
89/2	44	5.0	16.0	19.0	21.0
	82	2.0	2.5	--	28.0
	108	1.5	5.0	7.0	13.0
	111	7.0	20.0	23.0	23.0
	127	1.5	19.0	25.0	16.0
108B/3	44	--	17.0	17.0	25.0
	82	--	5.0	13.5	16.0
	108	--	5.5	11.0	16.0
	111	--	9.5	24.0	27.0
	127	--	14.0	22.0	29.0
AB	44	--	15.5	21.0	21.0
	82	--	2.3	11.5	15.0
	108	--	2.0	6.0	15.0
	111	--	12.0	26.0	27.0
	127	--	18.0	27.0	24.0

Only Myxococcus fulvus 44 and Chondrococcus blasticus 111 developed at all on the control, and then very poorly. On the three concentrations of cell suspensions the rates of growth of almost all of the organisms corresponded closely to the increase in number of cells in the suspensions. The colonies were smallest, with one exception, on the medium containing 25 milligrams of dried cells per 100 cc. That one exception was M. fulvus 44 on the Sarcina suspension,

108B/3, and the growth rate here was the same as on the next higher concentration, i.e., 50.0 milligrams per 100 cc. Differences between growth on the 50.0 and 100.0 milligram suspensions were less marked, in many instances, some species growing less rapidly on the highest concentration. This, however, might be attributed to any one of several causes such as the size of the inoculum, which is difficult to control exactly, the manner in which it happened to be placed on the medium during the inoculation (whether it was left as a spherical fruiting body or smeared out somewhat over a larger area), etc. The fact that most of the colonies on the suspension containing 100.0 milligrams of cells were equal to, or larger than, those on the 50.0 milligram media would indicate that, on the average, growth corresponded to the number of cells present.

No particular cell suspension appeared to be more favorable than any other as far as growth rates were concerned. Fruiting body production on suspension 108B/3 was somewhat better, in the highest concentration, than on the other two cell suspensions. In view of the succeeding experiment this does not appear to be significant.

The experiment was repeated using the same cell suspensions and the same species of myxobacteria. The incubation period was extended to fourteen days and the plates held at room temperature. More of the cultures produced

fruiting bodies over the longer period of time, but the relationships appeared to remain the same. In every case growth on the control was poor; only Podangium erectum 82 produced fruiting bodies on the 00.0 concentration after 14 days. All of the species fruited on at least one of the other three media, while Myxococcus fulvus 44 and P. erectum 82 developed fruiting bodies on every concentration of each cell suspension. There was no significant difference between the results on the three types of cells used for the suspensions, but the cell concentrations had a direct effect on rates of growth in almost every case. The difference between the two higher concentrations, 50.0 and 100.0 milligrams per 100 cc. of agar, was less marked than that between the lower concentrations. It would appear that in general 50.0 milligrams of cells in 100 cc. of medium is enough to supply the needs of the myxobacteria under consideration.

In a paper read before the annual meeting of the Society of American Bacteriologists, Snieszko, McAllister and Hitchner (1941) suggested a relationship between certain species of the Myxobacteriales and certain gram-negative Eubacteriales. At about the same time that problem happened to be under consideration by the present writer. Early examination had shown most of the associated

forms to be gram-negative, and the question naturally arose as to whether or not there was a definite relationship. Since many of the soil forms are gram-negative it would be quite understandable if such were the case.

The first pair of bacteria chosen for the test, that is to be used for the cell suspensions, were Bacillus subtilis and Escherichia coli. These were employed because both were relatively common and because of the fact that either or both might possibly constitute at least a part of a natural substrate. The cells were grown in large quantities and harvested as previously described. The dried and powdered cells were added to plain agar solutions in concentrations of 100 milligrams per 150 cc. of medium. This was sterilized in the usual manner. Plates were inoculated with the following myxobacteria:

Myxococcus fulvus 44

Polyangium fuscum 77

Podangium erectum 82

Chondrococcus blasticus 111

Myxococcus xanthus 127

Incubation was for ten days at room temperature. Readings, i.e., measurement of the diameters of the colonies, were made at two-day intervals. The results of the six-, eight-, and ten-day readings are shown in Table VIII, which also in-

cludes the results of a second experiment in which another pair of bacteria was employed for cell substrates. Again one gram-positive and one gram-negative species were used, but differing from the first pair in that the cells were pigmented. They were Sarcina sp. and Serratia marcescens.

Table VIII

Comparison of Suspensions of Gram-Positive and Gram-Negative Bacteria 10 Days at Room Temperature.

Age in Days	Cult. No.	Cells in Suspensions			
		Bacillus subtilis	E. coli	Sarcina sp.	Serratia marcescens
6	44	23.0	24.0	20.5	17.5
	77	9.0	13.0	6.0	13.0
	82	20.0	26.0	30.0	33.0
	111	26.0	27.0	20.0	26.0
	127	23.0	26.0	12.0	26.0
8	44	30.0	32.0	28.0	23.0
	77	15.0	19.0	7.0	24.0
	82	40.0	45.0	46.0	50.0
	111	34.0	36.0	25.0	34.0
	127	33.0	37.0	22.0	37.0
10	44	36.0	39.0	32.0	24.0
	77	17.0	22.0	7.0	31.0
	82	57.0	64.0	66.0	60.0
	111	38.0	42.0	29.0	36.0
	127	43.0	45.0	31.0	47.0

There is shown to be a slight increase in the rates of growth of all the species used, with the exception of M. xanthus 127, on the suspension of E. coli over that on the suspension of B. subtilis. However, the difference is so small that it is doubtful whether it could be considered significant or not. The same is true in the case of the suspensions of pigmented bacteria. The outstanding exception here is the growth of Polyangium fuscum 77 on the suspension of S. marcescens; this is much superior to that on Sarcina sp. However, the growth of this particular organism has been observed, in the past, to be more or less erratic so that this difference by itself could not be considered as of very great significance. The fact that it developed more rapidly on the non-pigmented gram-negative suspension than on the gram-positive, coupled with this large difference in rate of growth on the pigmented strains might have some bearing on the matter. If there is any difference between growth of these species of myxobacteria on gram-negative and gram-positive bacterial suspensions the data seem to point toward the gram-negative cells as the more favorable substrate.

The effect of pigmentation, too, is rather doubtful. While many of the colonies reached a somewhat greater size on the suspensions of white cells, the actual difference between rates of growth is small. Table VII showed fruiting body production to be best on suspensions of Sarcina while

in the present case it appears to be favored by suspensions of Bacillus subtilis. In general the formation of fruiting bodies apparently parallels, more or less directly, the concentration of cells in the medium; the kind of cells used in the suspension is of lesser importance.

DISCUSSION

The frequent growth on dung of the species of the Myxobacteriales that have been studied appears to be due, primarily, to the high bacterial content of the dung. If the water soluble constituents of dung were necessary for growth much better development might be expected on dung decoction agar than was actually observed. This medium produced much poorer growth than even nutrient agar. The constituents utilized by the myxobacteria would obviously seem to be the water insolubles, including fecal types of bacteria. These were utilized through the agencies of bacteriolytic enzymes.

Peptone, recommended by at least one investigator as necessary for good growth, seems to have a slight, but definite, inhibitory effect when used in combination with suspension of true bacterial cells. The nature of this inhibitory action is not known, but it might affect either the myxobacterial cells themselves directly, preventing the production of enzymes, in part, or it might act on the enzyme itself, once it had been produced, partially or wholly

destroying its activity.

In general, the kinds of associated true bacteria, acting as host cells to the myxobacteria, seem to have much less effect than the quantities in which they are present. In the absence of all other nutrient materials, with the exception of agar (which is able to support poor growth of certain species), the addition of such small amounts of dried cells as 10 milligrams per 100 cc. of medium resulted in a very definite increase in the rates of growth of all the organisms that have been studied. More than 50 milligrams of dried cells per 100 cc. of medium produced but small increases in growth rates. Under laboratory conditions this concentration of cell material seems to be optimum, rates increasing proportionally, up to this point, with the increase in concentration of cell suspension. It is possible that under natural conditions a higher concentration of host cells would be required for normal growth. The competition of the many types of soil forms would conceivably have some effect on the growth of the myxobacteria, increasing the nutrient requirements.

The gram reaction and pigmentation of the host cells may be minor factors in their utilization, though all of the myxobacterial species used in this work were able to lyse and develop upon all of the associates in the various

suspensions. It is possible that gram-negative, non-pigmented bacteria are more easily utilized by the slime bacteria, and inasmuch as such forms seem to predominate in the soil this preference could be understood. However, the myxobacteria are not especially fastidious, relative to the kinds of host cells, and appear to flourish in the presence of any of the Eubacteriales. This relationship seems to be so definitely one of dependence that it is considered as parasitic, rather than symbiotic.

SUMMARY

The relationship of the Myxobacteriales and Eubacteriales is discussed, and it is suggested that it is one of parasitism rather than strict symbiosis or association. Cells of the true bacteria are necessary for good growth and normal development of fruiting bodies by the myxobacteria. The host cells are destroyed by an extracellular lytic enzyme produced by the myxobacteria.

Myxobacterial growth rates and fruiting body production increase proportionally with the concentrations of killed bacterial cells in the medium, up to about 50 milligrams per 100 cc. of medium; above that point increases in rates of growth are less marked.

The evidence indicates a possible preference on the part of the myxobacteria for gram-negative, non-spore-forming, non-chromogenic bacteria, although this is not emphasized.

EXPLANATION OF FIGURES

Figs. 1 - 6. Comparison of the growth of Myxococcus fulvus, M. virescens, Chondrococcus blasticus and Podangium erectum on cell suspension media with that on nutrient agar and dung decoction agar over a period of 7 days.

Fig. 7. Fruiting bodies of M. fulvus produced on a host colony. The vegetative myxobacterial colony may be seen to extend beyond the margin of the host colony, and to have formed smaller fruiting bodies directly on the agar. 10 X.

Fig. 8. M. fulvus fruiting bodies formed on a different type of host colony. Note the tendency of the fruiting bodies to be formed on the eubacterial host colony rather than on the agar. 10 X.

Fig. 9. Pure culture of M. fulvus growing on a rabbit dung plate. The fruiting bodies shown are on the surface of the agar around the imbedded piece of dung. 20 X.

Fig. 10. Myxococcus virescens growing on and around a colony of true bacteria. 10 X.

Fig. 11. An immature fruiting body of Chondrococcus blasticus n. sp. The secondary fruiting bodies are shown developing from the large primary. On sterilized rabbit dung. 50 X.

Fig. 12. Mature fruiting body of C. blasticus. The large primary fruiting body has been completely utilized in the formation of the secondaries. 50 X.

Fig. 13. Several stages in the formation of cysts of Polyangium fuscum. The fruiting body first forms as a mass of colorless slime containing the long, flexible, rod-shaped bacteria. The cells then begin to group themselves at various points to form cysts, which become differentiated within the slimy mass. An early stage is shown near the upper left corner of the illustration; the cyst wall has not yet formed. Near the opposite corner are cysts in a more advanced stage: the cyst wall forming, but as yet un-pigmented. Cysts near the center are nearly mature and show large pigmented areas in the walls. 125 X.

Fig. 14. Mature cysts of P. fuscum. These are much larger than those shown in Fig. 13, and the pigment is evenly distributed throughout the walls. The slime envelope, which holds the mass of cysts together, is shown in both photographs. About 150 X.

Fig. 15. Clumps of fruiting bodies of Podangium erectum growing on agar. Oval or club-shaped cysts may be noted. 50 X.

Fig. 16. Cysts of P. erectum cleared in xylol and mounted in immersion oil. The moderately thick cyst walls may be seen, as well as the shortened, rod-shaped cells within. 430 X.

Fig. 17. Five day old colony of C. blasticus on bacterial cell suspension agar showing the translucent "window", or area of lysis, in the opaque medium. Fruiting bodies appear as small white dots. About 2 X.

Fig. 18. Lysis of cells in agar suspension by M. fulvus. The lysed area is nearly transparent whereas beyond the margin of the colony the medium is quite opaque. Fruiting bodies are seen to be formed in irregularly concentric circles. About 2 X.

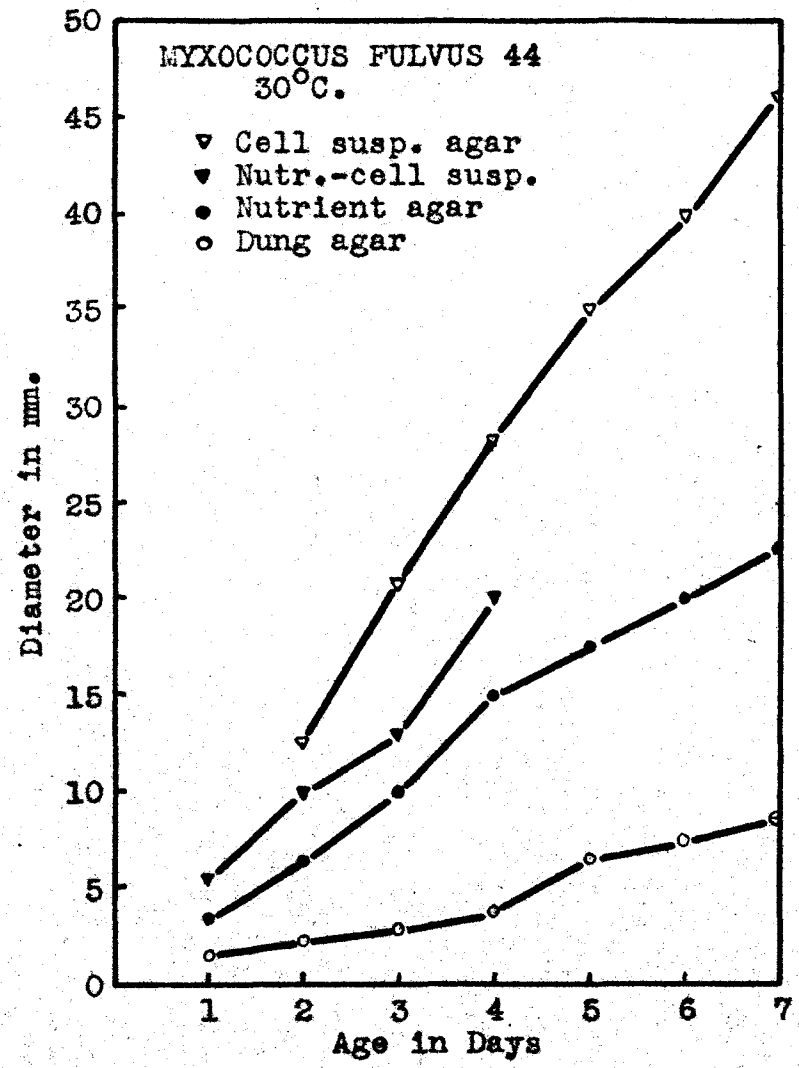
MYXOCOCCUS FULVUS 44
30°C.
Cell susp. agar
Nutr.-cell susp.
Nutrient agar
Dung agar
Diameter in mm.
Age in Days

Fig.1.

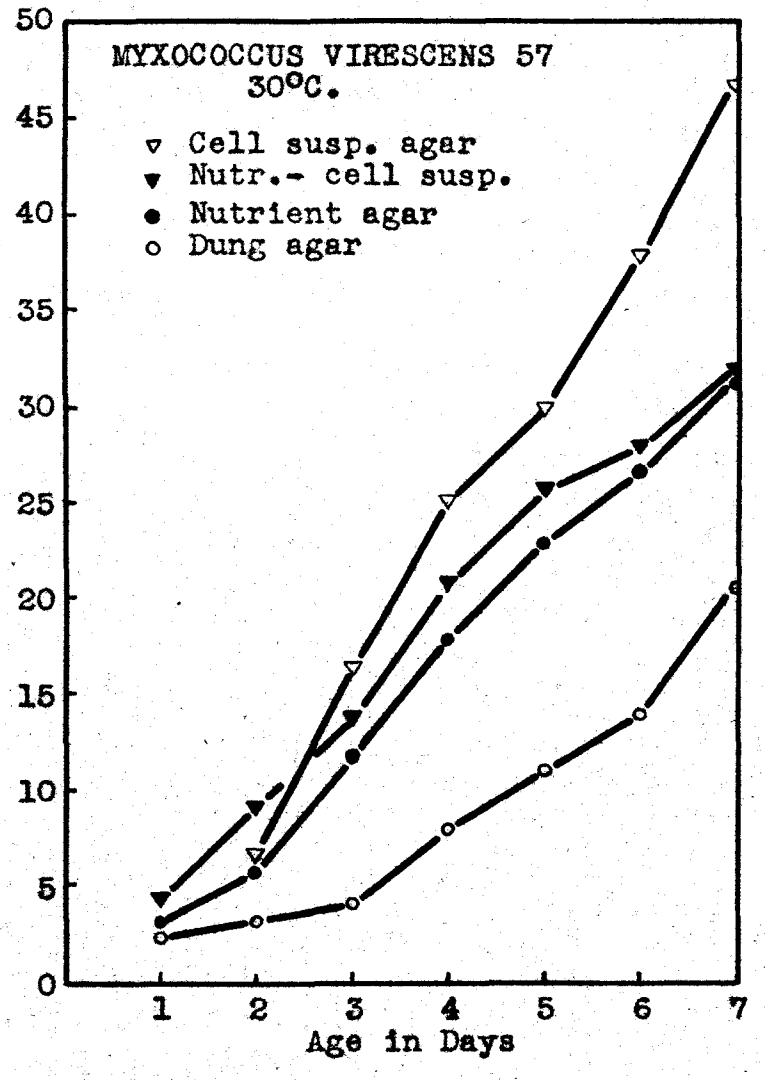
MYXOCOCCUS VIRESCENS 57
30°C.
Cell susp. agar
Nutr.- cell susp.
Nutrient agar
Dung agar
Age in Days

Fig.2.

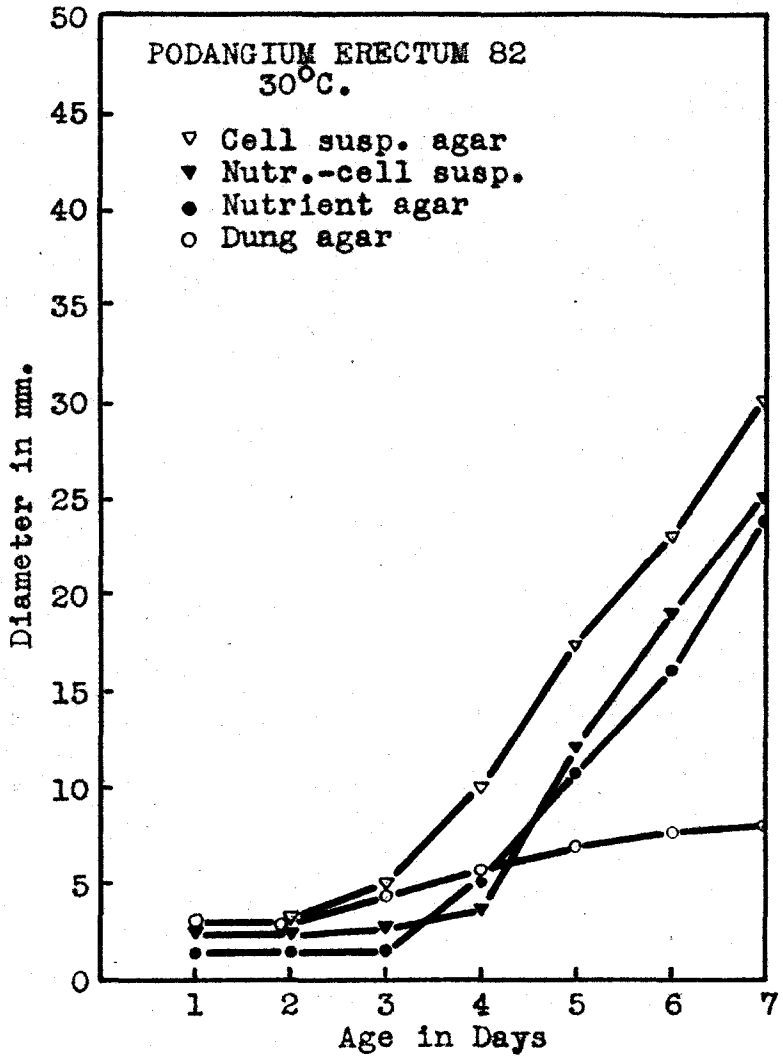
PODANGIUM ERECTUM 82
30°C.
▽ Cell susp. agar
▼ Nutr.-cell susp.
● Nutrient agar
○ Dung agar
Diameter in mm.
0
5
10
15
20
25
30
35
40
45
50
1
2
3
4
5
6
7
Age in Days

Fig.3.

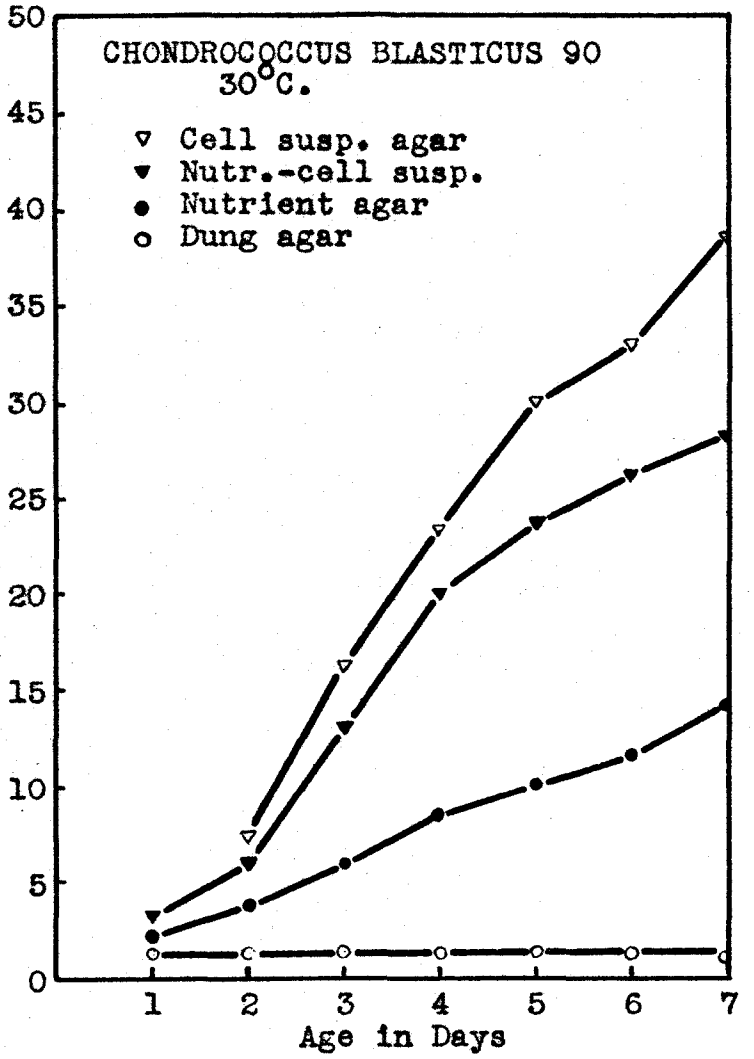
CHONDROCOCCUS BLASTICUS 90
30°C.
▽ Cell susp. agar
▼ Nutr.-cell susp.
● Nutrient agar
○ Dung agar
0
5
10
15
20
25
30
35
40
45
50
1
2
3
4
5
6
7
Age in Days

Fig.4.

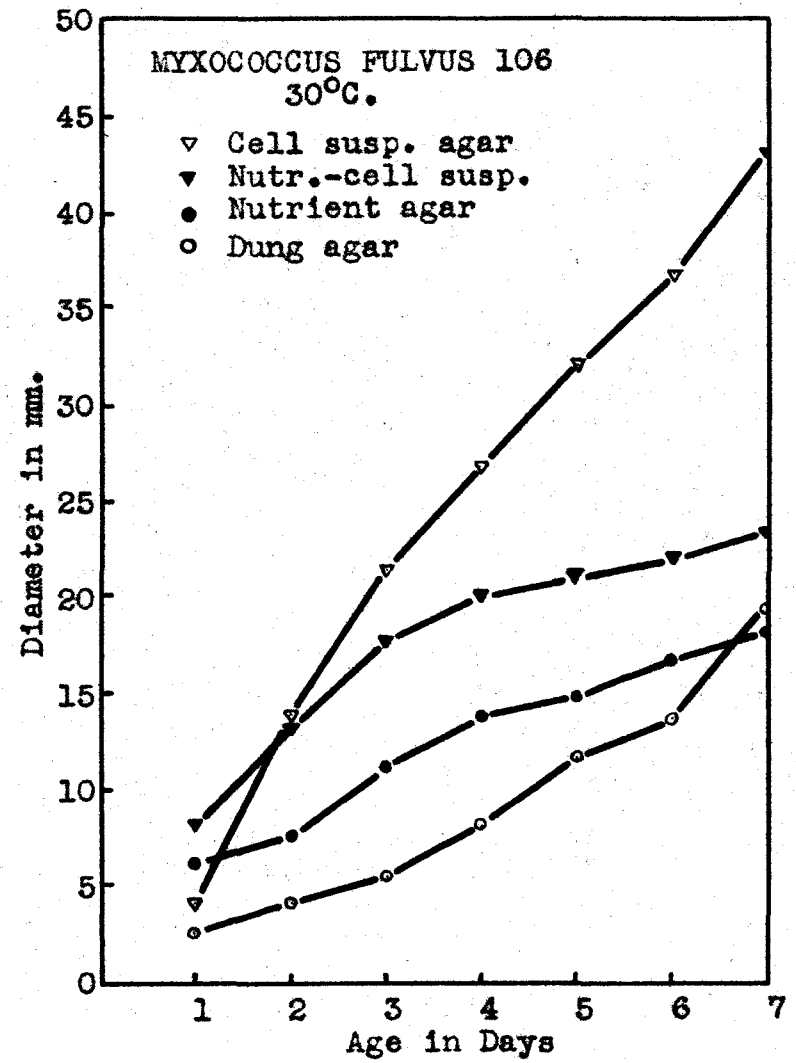
MYXOCOCCUS FULVUS 106
30°C.
Cell susp. agar
Nutr.-cell susp.
Nutrient agar
Dung agar
Diameter in mm.
0
5
10
15
20
25
30
35
40
45
50
1
2
3
4
5
6
7
Age in Days

Fig.5.

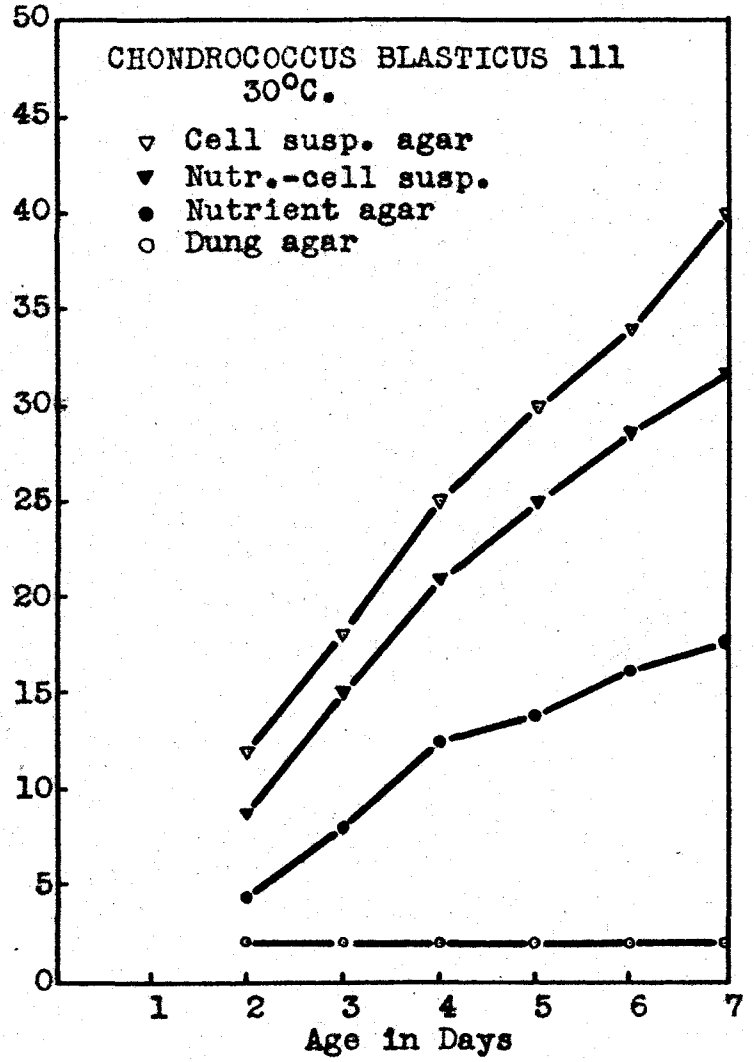
CHONDROCOCCUS BLASTICUS 111
30°C.
Cell susp. agar
Nutr.-cell susp.
Nutrient agar
Dung agar
0
5
10
15
20
25
30
35
40
45
50
1
2
3
4
5
6
7
Age in Days

Fig.6.

Fig. 7

Fig. 8

Fig. 9

Fig. 10

Fig. 11

Fig. 12

Fig. 13

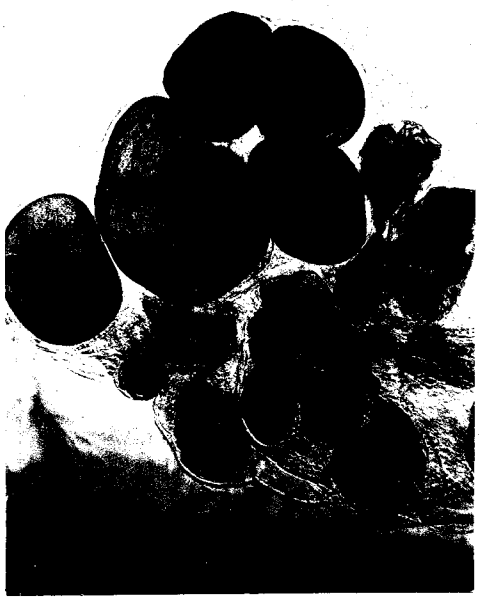

Fig. 14

Fig. 15

Fig. 16

Fig. 17

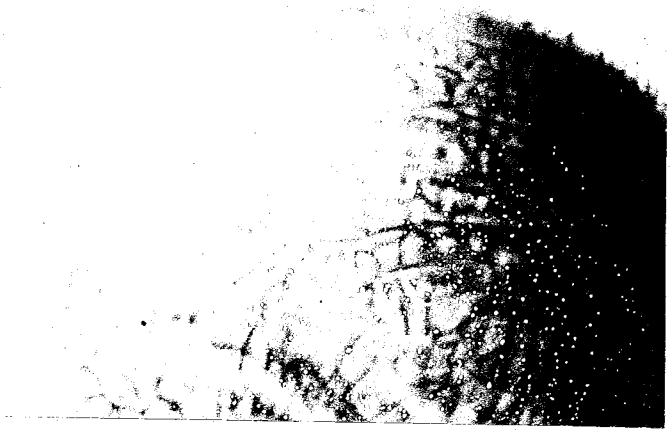

Fig. 18

III. THE MORPHOLOGY AND CYTOLOGY OF MYXOCOCCUS XANTHUS SP.N[1]

Knaysi (1938) states that there are three current points of view relative to the presence and nature of the bacterial nucleus. One group of investigators holds that the bacterial cell contains no nucleus. Knaysi argues, however, that the fact that a nucleus has not been seen is no indication that it does not exist. A second group maintains that nuclear material is present but in a highly dispersed condition and is therefore not easily seen. The third group takes the stand that the presence of a nucleus is logical, and is indicated by granular bodies often noted within the bacterial cell. These bodies are frequently observed to behave both functionally and chemically as nuclei are supposed to behave. In general, this latter view, that a more or less definite nucleus does exist, at least during certain stages of the life cycle of the bacterial cell, seems to be favored by many investigators today.

Stoughton (1929) observed the presence of stainable bodies in the cells of Bacterium malvacearum. Just before cell division occurred these bodies split, the halves

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[1]Now in press, Journal of Bacteriology, 1941.

migrating toward opposite ends of the cell, and finally were included in the new daughter cells. The dye reactions of these intracellular bodies were typically nuclear, and the author concluded that they should be considered as nuclei.

Much of the work published by the Hollandes (1930, 1932) favors the theory of a compact nucleus. Lindegren and Mellon (1932) described a type of reduction-division that took place within bacterial cells during certain phases of the life cycle. Badian (1935) stated that the condensed nuclear material to be seen in Bacillus megatherium may be observed to divide and rearrange itself in a manner quite suggestive of autogamy. Brooke (1936) studied a number of pathogenic bacteria and from his observations concluded that the granules he noted were composed of nuclear material. Allen, Appleby and Wolf (1937), working with a spore-forming bacillus, noted deep-staining bodies within the spores as well as within the vegetative cells. These bodies went through a rearrangement process after spore formation had taken place, forming recognizeable structures of various shapes. They contended that the spore has some function other than merely acting as a resting stage during unfavorable periods of growth. Rosca (1937) described a type of nuclear activity that he

considered asexual in certain cases, sexual in others. Although he did not actually observe the conjugation of two cells he could distinguish two types of chromosomes and considered them to be ♂ and ♀.

Chance's (1938) observations on Bacillus mesentericus disclosed a reorganization and redistribution of chromatin comparable to a mitotic division in higher forms, and Allen, Appleby and Wolf (1939) gave evidence of meiosis having taken place in a species of Bacillus which they studied. These same investigators also witnessed and accounted for at least two methods of spore formation in the one organism, and methods of reproduction other than the usual transverse fission. Lewis (1940) concluded that the theory of a more or less condensed nucleus, or at least a chromatin-incrusted gene string, is preferable to the theory of a diffuse nucleus. This worker pointed out that hypotheses of diffuse nuclei are not in keeping with findings on the transmission of hereditary characteristics.

Many of the publications on this subject deal with observations on spore-forming organisms. Possibly this is true because such organisms appear to undergo more marked cell reorganization just prior to, and during, spore formation. To the author's knowledge only two papers dealing with the cytology of myxobacteria have appeared. Krzemieniewski

(1928) studying cells of Sorangium sorediatum, Polyangium fuscum and of species of Myxococcus, Archangium, etc., and Badian (1930) working with Myxococcus virescens observed deeply-stained granules within the cells. These structures underwent a type of mitosis during the vegetative period of growth, and just before, and during, the process of sporulation displayed great variety of forms. Badian observed these intracellular structures to double in number, and then decrease by means of chromatin rearrangements. This was interpreted as a reduction-division, the whole process being considered a type of autogamy.

The present study began with the isolation and purification of a previously undescribed species of Myxococcus. In a number of ways this organism appears to be closely related to Myxococcus virescens (the species studied by Badian), particularly in the size of the vegetative cells and spores. These are larger than those of most of the other species of this group that have been observed in this laboratory. Because of the large size of the cells and the remarkable intracellular figures (one of the first things to be noticed), the species seemed to be well-adapted for cytological study. The species has been separated from M. virescens on the basis of the pigmentation of the fruiting body, a characteristic often used in the differen-

tiation of species of Myxobacteriales. The name Myxococcus xanthus is proposed for this organism. The diagnosis is as follows:

MYXOCOCCUS XANTHUS

Etymology: Greek (adj.) = orange, golden.

Diagnosis:

Fruiting body. Spherical to subspherical, usually sessile but occasionally constricted at the base giving the appearance of a short stalk or foot. Mature fruiting body 300 to 400 microns in diameter, often slightly flattened on top or on one side. Color varies from light yellowish-orange when young to bright orange when mature; color constant, never tending toward greenish yellow. No outer cyst wall or membrane discernable, the spores being imbedded in the slime holding the mass together. Usually single, though two or three fruiting bodies may become joined to form an irregular mass; each is attached to the substrate, however, and never bud, one from another.

Spores (Resting cells). Spherical, with thick outer wall or membrane. Highly refractile when unstained. Stain very easily with any of the ordinary bacterial or nuclear dyes. 2.0 microns

in diameter, sometimes slightly larger.

Vegetative colony (Pseudoplasmodium, swarm). The characteristics vary with the substrate. On plain 1.5 per cent agar (no nutrients added): Very thin and transparent, often hardly visible except by reflected light. Little or no pigmentation. Surface covered with fine, more or less regularly spaced, ridges, causing a dull macroscopic appearance without gloss or sheen. Margin thin and quite irregular. On rabbit dung decoction agar: Colony thicker, the surface being broken by veins or ridges radiating from the center. Thick center area often smooth and glossy while the margin appears much the same as that on plain agar. Veins or ridges extend outward in a loose spiral, always in a clockwise direction. Pigmentation, yellow to pale orange, is confined to the thicker central area, extends part way along the veins to the margin. On nutrient agar: Growth not good. Colony thick, at first heavily veined, the veins later merging to form an irregular glossy surface. Colony remains small, pigmentation usually fairly heavy; margin thick, irregular to lobate.

Vegetative cells. Large, flexible, single, gram-negative rods with rounded ends. No flagella, but move on the surface of a solid or semi-solid medium with a crawling or creeping motion. Vary in size from 0.5 microns to 1.0 microns by 4.0 microns to 10.0 microns; average 0.75 x 6.0 microns. More or less distinct cell wall often evident.

Found growing on dry cow dung from a pasture near Ames, Iowa.

METHODS

The best growth of M. xanthus, as with all of the myxobacteria that have been studied in this laboratory, is to be had on a solid or semi-solid medium with some source of the more complex carbohydrates. The work of the Krzemieniewskis (1926) has shown rabbit dung to contain all of the necessary factors for most of the myxobacteria. Due to the methods used in making the slide preparations in the present study, rabbit dung did not lend itself particularly well to the process except in combination with other methods. While the rabbit dung plates described below were not developed with cytological work in mind, they proved to be satisfactory for the vegetative and spore stages of the life cycle. Rabbit dung decoction agar was

employed for studying the germination of the spores.

Incubation was, for the most part, at room temperature. The work was carried on largely during the summer months and the laboratory temperatures of 22° C. to 27° C. proved to be satisfactory. Temperatures above 30° C. were not beneficial. At room temperature germination occurred in about 24 hours.

Culture methods. Rabbit dung plates were prepared by placing two or three pieces of rabbit dung in each petri dish and sterilizing, with the lids on, for one hour at 15 pounds pressure. When cool the plates were removed from the autoclave and allowed to stand over night in order to dry somewhat. Plain 1.5 per cent agar (no nutrients added) was then prepared. This was melted and sterilized in the autoclave for 20 minutes at 15 pounds pressure. When cooled to about 60° C. enough of the liquid agar was added to each plate to fill it to a depth of about one-half the diameter of the rabbit dung. This required 15 to 20 cc. of agar. Care was taken not to cover the dung with agar. Before the agar had a chance to harden the pieces of dung were moved to the center of the plate with a flamed needle. When the agar had set the plates were inverted. Inoculations were made by transferring one or two fruiting bodies from the stock cultures to the dung at the level of the surface of the agar. Diffusion of the

water soluble parts of the dung into the surrounding agar made some growth possible on the surface of the latter. Microscopic slide preparations were made from the colonies that developed on the agar around the imbedded pieces of dung.

Dung decoction agar was used in studying germination. This substrate was made by adding one liter of distilled water to 100 grams of dry dung. The mixture was heated to boiling and then allowed to infuse at room temperature for 24 hours. The solid material was then filtered off, and the filtrate made up to one liter by the addition of distilled water. Fifteen grams of agar were added and the solution sterilized in the autoclave for 30 minutes at 15 pounds pressure. When cooled to 60° C. plates were poured.

A fairly heavy suspension of spores was then made by transferring a few fruiting bodies to a sterile slide, inclosed in a sterile petri dish, on which a drop or two of sterile water had been placed. The fruiting bodies were broken up with a flamed needle. Loopsful of this suspension were then transferred to various points marked on the dung decoction agar plates. Incubation was at room temperature and slides were made at 12-, 24-, 48-, and 72-hour intervals.

Methods of slide preparation. Originally an attempt was made to study the cells of M. xanthus by employing the usual type of smear technique. This was found to be entirely

unsatisfactory. It was impossible to spread the material out on the slide, once it had been stripped from the agar substrate, due to the membranous nature of the colony. Those few cells that had been teased away from the mass were often so badly bent and distorted that the size and shape were questionable. Also, the fact that no indication of the dispostion of the cells on the colony was to be had by this method made it imperative to use some other means.

Cover slip preparations were finally decided upon as being the most satisfactory. For this purpose number one cover slips, seven-eighths inch square, were used. These were cleaned well and stored in alcohol. Just before use they were rinsed in 95 per cent alcohol and flamed. This was repeated twice. After the final flaming the cover slip was placed, by means of sterile forceps, over that section of the colony to be studied, and pressed down carefully to assure good contact. The only difficulty was with air bubbles, and it was found that with a little care these could be pressed out from under the glass. Generally the cover slips were left in place for about two minutes, though the time factor was not critical. The cover slips were then removed with sterile forceps, care being taken to raise the glass, not to slide it. Part of the plan was to obtain a picture of cell distribution and any sliding motion would, of course, disturb the location of the bacteria.

When dried the cover slips were placed in 95 per cent ethyl alcohol for fixation. A fixing time of three minutes was found to be satisfactory. Various mixtures of alcohol and xylol were also tried as fixatives but no improvement could be noted over the alcohol alone. After fixation the slides were allowed to dry in the air.

Staining methods. (1) Gentian violet-iodine: As developed, this method was a modification of the gram technique. The fixed preparations were stained for five minutes in anilin oil-gentian violet, rinsed in distilled water, and then mordanted five minutes in Lugol's solution. This was followed by another wash in distilled water. The preparation was deeply stained, dark blue in color, with little differentiation between the various parts of the cell. A brief rinse in 50 per cent alcohol was enough to decolorize the non-chromatic parts of the cell and show up the nuclear structures. Since the cells are decidedly gram-negative, decolorization was necessarily brief. Counter staining was found to be superfluous and was dispensed with. Decolorization was stopped by a final wash in distilled water, and the preparations were dried in the air and mounted in neutral balsam.

(2) Iron-hematoxylin: The fixed and dried preparations were mordanted in a 4.0 per cent solution of iron-alum for two hours, washed in distilled water, and placed in a well-

ripened 1.0 per cent solution of hematoxylin (in 10.0 per cent alcohol) for two hours. This was followed by a rinse in distilled water. When differentiation was thought to be necessary, a 1.0 per cent solution of iron-alum was used. Differentiation was less necessary in the case of this dye, however, than in the previous one, and for the most part was dispensed with. Counter staining was not employed.

(3) Feulgen's stain: The Tomasi modification as described by Conn (1936) was followed. The fixed and dried preparations were given a brief rinse in cold 1/N HCl, placed in another 1/N HCl rinse (at 60° C.) for four minutes, and then rinsed again in cold 1/N HCl. This was followed by a wash in distilled water. The preparations were then placed in a solution of decolorized basic fuchsin and allowed to stain for two hours. Decolorization was in an acidified potassium metabisulphite solution. After rinsing in distilled water and drying, the cover slips were mounted in neutral balsam. Counter staining was not found to be practical.

The quality of the basic fuchsin used in this test is of prime importance. The first dye lot that was tried gave very poor results; another lot, certified for the particular purpose, was found to be satisfactory. Checks were run on slides of onion root tip in order to make sure that the procedure, as well as the dye, was correct before attempts were

made to stain bacteria.

(4) Other stains: A number of other dyes, for the most part the usual bacterial dyes, were tried in connection with this study. The majority of them were found to be unsatisfactory. Spore stains such as the Ziehl-Neilsen stain, the malachite green stain, etc., gave negative results on both spores and vegetative cells of M. Xanthus. Both types of cells decolorized rapidly and completely. However, some of the differences between the spores of this organism and those of the true spore-forming bacteria were emphasized. Gram's stain gave negative results. Loeffler's methylene blue, while satisfactory in part, gave results inferior to those obtained with gentian violet and iron-hematoxylin. Safranin and carbol fuchsin were not satisfactory. Aceto-carmine and iron-acetocarmine were expected to give good reactions but rather poor results were had with both. Sudan III was tried in an attempt to show that the vacuoles occasionally seen within the cells were of fatty materials, but without success, and iodine in the form of Lugol's solution and as a dilute alcoholic solution was used as a test for glycogen. It gave negative results in the case of the vegetative cells, doubtfully positive in the case of the spores.

Forty-five millimeter petri dishes were employed for all procedures that required more than a few minutes for

mordanting and staining. These were more satisfactory than larger containers since the small preparations could be processed separately.

MORPHOLOGY AND CYTOLOGY

The germination of the spores, or resting cells, might be considered as the beginning of the life cycle of M. xanthus, but the vegetative phase appears to be the dominant one; for that reason the vegetative phase is considered first. The vegetative cells of Myxococcus xanthus are long, flexible, gram-negative rods with rounded ends (Figs. 1, I - L; 9 - 11). They have no flagella but appear to move by means of a crawling or creeping motion on top of the layer of slime with which they pave the substrate. The exact nature of this motion is not well understood, but it has been suggested to be the result of an asymmetric excretion of slime by the cell, producing more pressure on one end than on the other. No wriggling motion such as is usually associated with motile bacteria can be observed, but the cell can be seen to change its position, from time to time, in relation to some fixed point. Examinations of living preparations of M. xanthus have shown the cells to travel at a rate of about 7 microns per minute. The cells always move away from the center of the colony, but not in a straight line: there is always a curving of the path in

a clock-wise direction. This varies in intensity but may usually be noted to some degree.

The distribution of the cells on the colony as shown in Figs. 9 - 11 is characteristic not only of this particular species but of the genus. Very seldom is more than a single layer of cells observed on a colony. The cells are usually arranged in small groups of from two or three to a dozen or more, lined up with their long axes parallel, and moving in the same direction. The entire group moves as a unit, along a "front". While a few of the cells seem to be traveling independently the large majority always follow the same slightly curved path toward the margin of the colony. Generally they are not packed closely together, but are separated by about the diameter of one cell.

The vegetative cells average 6.0 microns in length by about 0.75 microns in diameter. Unstained the cells are long flexible rods, usually rounded on the ends. However, they occasionally appear to be slightly tapered at one or both ends, particularly those cells at the margin of the colony; very few are spindle-shaped. More often than not the younger unstained cells show several highly refractile granular bodies, varying in number from two to eight, while the older vegetative cells show one or two rather large refractile bodies at or near the center of the cell. The fixing and staining procedures previously

outlined appear to have very little effect on cell morphology, the only noticeable change being an extremely slight swelling at the ends of the younger cells. This gives them the cylindrical appearance of the older cells.

The cells stain fairly well with most of the usual bacterial dyes, in some cases a thin cell wall being visible. The center of the cell is occupied by a deeply-staining body approximately one-third the length of the cell and equal in diameter to the cell; i.e., about 2.0 by 0.75 microns. As far as can be told at the present time this body has no limiting wall or membrane, but is rather a mass of compact nuclear material. It has a marked affinity for gentian violet and for iron-hematoxylin, and gives a positive reaction with Feulgen's stain. The remaining portions of the cell take all stains lightly. Occasionally the cell appears to contain vacuoles at one or both ends but tests for glycogen and for fats have failed to indicate the nature of the vacuolar material. Functionally the condensed body located at the center of the cells seems to be nuclear also. Vegetative reproduction is by means of transverse fission. Prior to cell fission a division of the nucleus takes place. Stages in this process may be seen in Figs. 1, I - L; 9 - 11. At first the nucleus enlarges longitudinally to almost twice its length and then begins to constrict at a point near its center, producing a dumbbell-shaped body. The halves finally pull

apart and migrate toward opposite ends of the cell. After nuclear division has been completed actual cell fission begins. This also is accomplished by constriction and not by the formation of a transverse wall. The steps in the process are shown in Figs. 1, I - L; 9 - 11. No case has been observed in which the daughter cells remain united in pairs after cell division has been completed. Each new cell contains the single, deep-staining, compact nucleus. Sometimes, during periods of rapid growth, a second nuclear division takes place before fission begins, and in such cases cells with four nuclei may be noted. These are not to be confused with a later stage in which four stainable bodies are typical.

The vegetative phase continues for 4 to 10 days, the time depending upon conditions of temperature and environment. At room temperature (21° - 25° C.) and on rabbit dung plates the first signs of fruiting body formation begin to be noticed after 5 to 6 days. Morphologically a slight increase in the diameter of the cells is the first indication of the change about to occur. (Figs. 1, M; 12). Most of the cells measure approximately 1.0 micron in diameter in this early transitional phase, and this increase in diameter may also be accompanied by a slight decrease in length. At first there is not much change in the internal structure of the cell, but soon the nucleus is noted to have broken up, and particles of deep-staining material are to be seen

throughout the length of the cell (Figs. 1, M; 12 - 16). During this stage the cells begin their migrations toward some predetermined "fruiting center" on the colony.

The stimulus for the gathering of the cells from within a given area for the express purpose of sporulation and the formation of fruiting bodies is not understood. No apparent change in temperature or environment is necessary although a lack of food material may play a part. More likely would be the production of metabolic products by the cells, or a slight change in the moisture content of the medium. At any rate, the motion of the cells toward the margin of the colony suddenly ceases, and all cells within a given area begin to move toward a central point. This is best shown in Fig. 17. As the bacteria approach this point the morphological and cytological changes taking place are particularly marked. Observations by several workers on species of Bacillus, and by Krzemieniewski (1928) and Badian (1930) on species of myxobacteria, have indicated that a number of changes occur within the cell prior to sporulation; whether, as in the case of species of the Bacillaceae, the spore is formed as a structure within the cell, or whether, as in the case of Myxococcus xanthus and other myxobacteria, the cell is the spore, rearrangement and redistribution of chromatin material appears to be a preliminary step in sporulation.

As they approach the location where the fruiting body is to be formed, or is in the process of formation, the cells increase in diameter and become perceptibly shorter (Figs. 1, N; 3, 14, 15, 16), and rearrangements in the chromatin material become quite apparent. The chromatin previously distributed throughout the length of the cell collects in four more or less distinct masses (Figs. 12 - 16). These bodies are not arranged within the cell in any particular fashion in the earlier stages, nor do they seem to have any particular shape although their outlines are sharp. In general, they give the cell a banded appearance; two of them are usually located at the poles, the other two near the center of the cell. At this stage the cell is about 1.0 to 1.5 microns in diameter by 4.0 to 5.0 microns in length. There is a good deal of variation in cell size during these phases, and cells as broad as 2.0 microns have been noted.

The next phase is typified by an elongation of the four structures into rod-shaped bodies or chromosomes. These become arranged in pairs with their long axes parallel to each other and to the long axis of the cell. One pair tends to locate toward each end of the cell. These rod-shaped chromosomes then begin to break up into chains of bead-like bodies that are interpreted as chromomeres. The break is not complete, however, for the chromomeres remain in a

chain, and under proper lighting can be seen to be held together by slender threads of chromatin material. There is little doubt that these bodies are chromosomal in nature; they stain readily with the nuclear dyes, particularly iron-hematoxylin, and seem to function as prophase chromosomes in cells of higher forms. The chromomeres are refractile, and were so photographed. It will be noted in Fig. 17 that those cells nearer the margins of the illustration are in slightly different focus and show the chromomeres as deeply stained bodies within the cells. The number of chromomeres to each chromosome is difficult to determine, though total counts, i.e., the total number of chromomeres per cell, varied up to about 28. This would indicate a maximum of seven to each chromosome, if they were divided evenly between the four structures. In this stage the chromosomes are still typically in pairs, one toward each end of the cell. This stage appears to be of brief duration since having been observed only a few times during the examination of 350 to 400 slides, but it is thought to be typical. The illustrations, Figs. 17 and 18, were made from a slide in which nearly all of the cells were in this particular stage and afforded a good opportunity to study the structures. In all other cases only a few cells in the "prophase" stage were to be noted, and these more or less isolated from each other. In appearance, however, they were all very similar.

To this author's knowledge this is the first time any structures resembling prophase chromosomes have been observed in bacteria of any kind.

These long chain-like bodies soon shorten to oval-, rod-, or comma-shaped structures (Figs. 1, P; 2, K; 3, 23, 24, 25, 26) and appear to undergo a type of autogamous fusion. The cells, in many instances, are nearly spherical, often 2.5 to 3.0 microns in diameter or larger, allowing the chromosomes to assume almost any position within the cell. In some cells they are seen to be paired, some of these being shown in Fig. 3, while in other cells only two large bodies are to be noted, indicating a fusion has occurred. This corresponds to the phenomenon described by Badian (1930) as taking place in Myxococcus virescens and considered by that author as being autogamous fusion.

During the fusion of the pairs of chromosomes, to form a binucleate cell, the size of the cells may be seen to begin to decrease (Figs. 23, 24), and the staining reactions to become more marked. Often it is hard to differentiate the internal and external parts of the cell. The cell wall begins to thicken and stain more deeply (Figs. 1, Q; 20 - 27) and the two chromatic bodies, or nuclei, if not actually joined together, become closely appressed as though about to unite to form a cell with one large nucleus. It is thought

that this actually does take place either during spore formation or while the cell is in the so-called resting stage. The latter assumption appears to be possible. Allen, Appleby and Wolf (1937) noted marked changes in nuclear structures in the spores of Bacillus sp. while the cell was supposed to be in a resting condition. Further indication of nuclear activity during the matured, or nearly matured, spore stage of M. xanthus is the fact that germinating cells frequently show a single large nucleus in the process of division.

The ripe spore is a spherical cell almost exactly 2.0 microns in diameter. Unstained it is highly refractile, and has a thick, easily visible wall. The spore is highly receptive to all bacterial and nuclear stains that have been tried, giving, in most cases, a very dense, completely opaque preparation with no internal structures visible. It is definitely positive to Feulgen's stain, the entire cell becoming pink. This is not true of the previously described stages in which only the nuclear bodies gave a positive reaction. Gentian violet-iodine gives a dark blue, almost black, color, and iron-hematoxylin colors the spore black with no details visible. Cells so stained are shown in Figs. 16 - 24. Gram's stain shows the spores, like the vegetative cells, to be markedly gram-negative, but when a somewhat specialized

technique was employed, i.e., intense staining and mordanting and careful destaining, combined with dilute counter stain, one large gram-positive area within the gram-negative cell could often be seen. Whether this was due to incomplete destaining or not is uncertain. The fact that the gram-positive areas were not always centrally located might indicate some differences between their composition and that of the rest of the cell. If it were entirely a matter of incomplete destaining it would be reasonable to expect that the area would be centrally located since the entire cell wall is likely to be of equal permeability throughout. This would appear to support the assumption that the two nuclei in the immature spore combine to form a single large nucleus. By the time the cell reaches this stage it is imbedded in the slimy material holding the fruiting body together (Fig. 5).

The differences between the spores of M. xanthus and those of species of Bacillaceae are notable. Spore stains such as Ziehl-Neilsen and the malachite green stains that show a definite differentiation between the spore and the sporangium in the case of the true bacteria show no difference whatever between the spores and the vegetative cells of M. xanthus. Decolorization is complete in both cases.

On the other hand, stains such as Loeffler's methylene blue, gentian violet and the like, that ordinarily indicate the presence of a spore by a complete absence of color (with the ordinary techniques), produce a deep, opaque coloration in the spores of M. xanthus. Species of Bacillus generally form the spore within the cell, whereas in M. xanthus the spore is the cell, the entire structure of which takes part in sporulation.

Germination of the spores seems to occur only when a new environment is made available. It would hardly seem possible that the growth of a single layer of bacteria over the surface of the substrate and the eventual production of fruiting bodies would exhaust the food supply of that area. It is more likely that the various metabolic products formed by the cell, or an unfavorable change, or lack of change, in moisture content, might cause the environment to become unsuitable for germination. Once fruiting has taken place little, if any, more vegetative growth is to be seen in that given area. Attempts to promote germination on the same substrate on which fruiting took place failed. Transfer of some of the fruiting bodies to a fresh medium produces a vigorous new growth.

Studies on the germination of the spores were carried on by growing the cells on a dung decoction agar, rather than on sterilized rabbit dung imbedded in agar. Slide

preparations made after twelve hours incubation at room temperature showed no signs of any changes having occurred in the spores. At the end of a 24 hour incubation period at least half of the cells had begun the process of germination (Fig. 6) while some of them had completed it.

The first indication of a change was the lessened affinity of the cell for dyes (Figs. 1, B; 6 - 8). During the earliest germinal phases the internal structures of the cells are very difficult to observe (Figs. 1, A; 6 - 7) but shortly one or two stainable bodies are to be noted (Figs. 1, B; 4, 7, 8). There next appears a softening at some point on the cell wall, which has in general become much thinner, and a slight bulge appears. The stainable body, nucleus, migrates toward that point (Figs. 1, C; 4, 6, 7) and moves into the vegetative cell as the latter forms. Often the nucleus is to be seen as a dumbbell-shaped body during this stage (Fig. 4, C, D, E) and may take a position at the distal end of the newly germinating cell and remain there until germination is complete (Fig. 1, D - G). As a general thing nuclear division is complete by the time the cell has germinated, division being accomplished by constriction as in the case of the vegetative nucleus. The new binucleate vegetative cell (Fig. 8) is completely

freed of the spore wall when it has reached a length of 3.0 to 4.0 microns. Until one division of this binucleate cell has occurred it can hardly be considered a typical vegetative cell. This post-germinal division seems to take place soon after germination giving rise to two mononucleate vegetative cells. The new cells acquire the ability to move within a short time, and after 48 hours will usually have formed a colony large enough to see with the unaided eye.

DISCUSSION

The cells of Myxococcus xanthus go through a comparatively complex morphological cycle. In the vegetative (dominant) phase the cells are long, slender, flexible rods with rounded or slightly tapered ends. Multiplication is by transverse fission by means of constriction rather than by the formation of a transverse septum. The cells crawl over a layer of slime which they excrete on the substrate. Motion always follows a clockwise spiral path away from the center of the colony. The cells are often arranged in small groups which move as a unit.

After several days in the vegetative phase the cells begin to gather around various fruiting centers. Changes in morphology are apparent, particularly a shortening of

the cells. As they approach the point where the fruiting body is in process of formation the cells may become almost spherical. By the time they are incorporated in the slimy mass of the fruiting body the cells have become a perfectly spherical, thick walled, non-motile spore.

Germination occurs when the spores are placed in a new environment. The first indication of germination is a thinning of the cell wall. At some point on the wall a slight bulge appears. This develops, by means of a process somewhat similar to budding, into an elongate rod-shaped cell. When this cell has reached a length of 3.0 to 4.0 microns it becomes detached from the old spore wall by constriction at the point of emergence. After a single post-germinal division the cell is typically vegetative and begins the life cycle again.

Cytologically the life cycle of M. xanthus is correspondingly complex. In the vegetative phase a single large nucleus, occupying the central third of the cell, is to be seen. This body has a marked affinity for such nuclear stains as iron-hematoxylin and gentian violet, and gives a positive Feulgen reaction. There seems to be no limiting membrane inclosing the nucleus, but it is rather composed of a compact condensed mass of nuclear protoplasm. Its boundaries are quite definite. It seems to be granular

in structure and apparently contains the equivalent of one pair of chromosomes (Fig. 2, A). There is no evident arrangement in threads or chains such as might be found in the close spireme phase in the cells of higher plants. Nuclear division is by means of non-random amitosis. Prior to cell division the nucleus increases in size (Fig. 2, B), along with the increase in size of the cell itself. Each particle of which the nucleus is composed must double in size and split, the various halves migrating toward opposite poles of the cell. This corresponds to the splitting of chromosomes before a straight somatic nuclear division: each new cell receives its portion of chromatin material carrying hereditary genes. After enlarging the nucleus begins to constrict at a point near the middle (Fig. 2, C), the halves pulling toward opposite ends of the cell. Just before division has been accomplished, and before cell fission is obviously under way, the nucleus has the appearance of a dumbbell-shaped body (Fig. 2, C). This condition has been at times previously described as the normal condition of the nucleus; actually it is a stage of comparatively brief duration. Nuclear division is always completed before cell fission begins (Fig. 2, D); in a rapidly growing colony two nuclear divisions may take place before the cell itself has completed one division. Such cells appear to

have four nuclei. This condition is not similar to a later stage in which four bodies, chromosomes, are to be seen within the cell. The two should not be confused. Once nuclear division is complete the cell constricts at the middle (Fig. 2, E), producing two new mononucleate daughter cells. This type of vegetative reproduction continues for an indefinite period of time.

When the cells begin to converge upon a fruiting center definite changes in cell structure begin to be seen. The first noticeable change is an increase, slight in some cases, in the size of the nucleus (Fig. 2, F). The cell at this point is quite broad, and the nucleus, at first occupying a central position as in the typical vegetative phase, begins to spread out and become dispersed through the length of the cell; at this particular stage it might be considered diffuse (Fig. 2, H). Shortly, however, four masses of nuclear material are to be noted, each corresponding to a chromosome (Fig. 2, I). At first these are irregularly shaped masses, occupying various positions in the cell, but as the cell progresses toward the fruiting center the bodies within become more definite until four distinct structures may be seen. They elongate to rod-shaped bodies, pair up, and each chromosome breaks up into a chain of chromomeres (Fig. 2, J). In this stage the chromosomes are similar, if not identical, to the

prophase chromosomes in the cells of higher plants. Chemically and functionally these structures are chromosomal in nature. This stage is of brief duration, having been observed only a few times during the examination of numbers of slides, but it is thought to be a typical step in the mitotic division of the nucleus of M. xanthus.

Having become arranged in pairs the prophase chromosomes begin to shorten, becoming rod-, L-, or comma-shaped (Fig. 2, K). The cells are much thickened and shortened by this time, and the arrangement of these pairs of chromosomes varies considerably. No correlation to the long axis of the cells has been noted. Finally a union of the pairs of chromosomes is accomplished, producing the binucleate cell (Fig. 2, L), each nucleus containing the equivalent of one pair of chromosomes. As a rule the two nuclei are about the same size, although in some cases one may appear to be somewhat larger than the other. Rosca (1937) based his theory of sexual union on the pairing of two bodies unlike in size. It is thought that any difference in the size of these two nuclei in M. xanthus is purely incidental.

At this point the cell has become almost spherical, and the cell wall has begun to thicken. The spore is already imbedded in the fruiting body and must have lost all power of locomotion. No motile spores have ever been seen. It is quite probable that a second nuclear union takes place

at this time or after the spore has been formed. Due to the extreme affinity of the cell for dyes and the thickness of the cell wall it has been found impossible to observe changes in the nuclear structure during these phases. However, the division of a single large nucleus in the early stages of germination points toward a previous autogamous nuclear fusion (Fig. 2, M-M) and it is thought that the spore goes through the resting stage as a large single nucleus, diploid in nature, surrounded by a thick cell wall, and containing little, if any, other cytoplasmic material. That the spore is basically nuclear is shown by the dye reactions, particularly the definitely positive Feulgen reaction. No vacuoles of any sort have been noted within the spores, and the test for glycogen was questionably positive. Apparently little reserve food material is present. An occasional pre-spore stage cell may show one or two vacuoles, the remainder of the cell giving positive nuclear reactions. These cells are not often seen, however, and attempts to ascertain the nature of the vacuolar material were unsuccessful.

Germination of the spores is first indicated by a lessened affinity for dyes. At this time the large single nucleus initiates a pre-germinal division (Fig. 2, Q) that progresses as the spore germinates. By the time the newly emerged cell is ready to separate from the old spore wall

(Fig. 2, S) the nucleus has divided, producing a rod-shaped binucleate cell. Unless a chromosomal union has been effected during the spore stage this nuclear split is a reduction-division for the purpose of producing vegetative cells with the typical number of chromosomes. Following the division of the nucleus the cell elongates, constricts at the middle, and divides, producing two typical vegetative cells, each with a single compact mass of nuclear material in the center.

If any sexual union were to be noted it would of necessity occur immediately following the post-germinal nuclear division, resulting in four haploid cells. The union of a pair of these cells would be necessary for the formation of typical vegetative cells. However, no indication of cell conjugation has been observed, and it is thought that this step, which is typical of some of the fungi, is not included in the life cycle of M. xanthus.

SUMMARY

A new species of Myxococcus producing an orange-colored fruiting body is diagnosed and described, and the name Myxococcus xanthus proposed.

Methods of growing the bacterium on media utilizing rabbit dung as the source of nutrient material are described.

Microscopic slide preparations were made by pressing clean, sterile cover slips down on the growing colony. The adhering cells were fixed, stained and mounted in neutral balsam.

The life cycle of M. xanthus is relatively complex. In the vegetative stage the cells are long, flexible, single rods, that move over the surface of the substrate by a crawling or creeping motion. They are grouped on the substrate in small clumps, their long axes parallel, and move in clockwise paths as a unit toward the margin of the colony. As the cells go into the spore stage they shorten and become perfectly spherical by the time they are imbedded in the slime of the fruiting body. Germination is by a process analagous to budding.

Evidence is presented supporting the theory of a compact or condensed nucleus. In the vegetative phase the nucleus is a single compact mass of nuclear protoplasm that divides prior to cell fission. It has a marked affinity for nuclear dyes, and is Feulgen positive. During the transitional phase the nucleus breaks up into four chromosomes that are stained by gentian violet and iron-hematoxylin. In the prophase the chromosomes are shown to be made up of chromomeres. An autogamous fusion of chromatin material occurs before the mature spore has been formed, and nuclear

division, presumably meiotic, takes place during germination of the spores.

SECTION III

EXPLANATION OF FIGURES

Fig. 1. Reconstruction of the life cycle of M. xanthus. Originally drawn to a scale: 1 cm. = 1 micron. A, mature spore; B, early germinal stage; C - H, germination; I - L, vegetative phases; M, transitional; N - O, prophase; P, early sporulation stage; Q - R, sporulation; S, mature spore.

Fig. 2. Diagrammatic outline of nuclear divisions; not drawn to scale. A - F, vegetative phases; G - J prophase; K, late prophase; L, chromosomal fusion; M, nuclear fusion; N - O, spore, mononucleate, diploid; P - Q, early germinal, diploid; R - S, germination; T, binucleate vegetative; U, post-germinal division; V, haploid vegetative cells.

Fig. 3. Various stages in the life cycle (excluding germination) of M. xanthus. Drawn to scale: 1 cm. = 2 microns. Figures from preparations stained with iron-hematoxylin and gentian violet-iodine. Measurements by means of a Whipple eye piece.

Fig. 4. Stages in the germination of M. xanthus. Drawn to scale: 1 cm. = 1 micron. A, spore; B, early stage in germination: division of nucleus has already occurred; C, first stage in formation of new cell: nucleus dividing; D, later stage; E, germination nearly complete: nucleus dividing; F, newly germinated cell and old spore wall; G, binucleate vegetative cell before first division.

Fig. 5. Fruiting bodies of M. xanthus growing on rabbit dung. About 20 X.

Fig. 6. Germinating cells in various stages, Gentian violet-iodine. 1860 X.

Fig. 7. Same as Fig. 6. Iron-hematoxylin. 1160 X.

Fig. 8. Same as Fig. 7. Newly formed binucleate cells may be seen.

Fig. 9. Vegetative cells, showing typical distribution. Stages of vegetative reproduction may be seen. Gentian violet-iodine. 1860 X.

Fig. 10. Same as Fig. 9.

Fig. 11. Late vegetative stage showing some cells with four deeply stained bodies. Gentian violet-iodine. 1860 X.

Fig. 12. Late vegetative stage showing cells with nucleus breaking up into four bodies. Iron-hematoxylin. 1860 X.

Fig. 13. Late vegetative and early transitional stages. Cells with two and four chromosomal bodies. Iron-hematoxylin. 1860 X.

Fig. 14. Same.

Fig. 15. Same. Gentian violet-iodine. 1860 X.

Fig. 16. Various stages in spore formation. Gentian violet-iodine. 1860 X.

Fig. 17. Cells with chromosomes in the prophase stage. Migration of cells toward the fruiting center may be noted. Focus at the center is slightly different than at the margins of the illustration giving the chromatin material of the cells nearest a refractile appearance; cells near the margins show the chromosomes to be stained black with iron-hematoxylin. 1160 X.

Fig. 18. Enlargement of cells from the center of Fig. 17. This illustration appears negative as the final print was made from a dia-positive rather than from an additional negative. Chromosomes are deeply stained but were focused to show as highly refractile bodies since they are more easily seen; they thus appear black in the negative print. 3500 X.

Fig. 19. Various stages in sporulation. A few cells containing large vacuoles; otherwise nuclear material. Iron-hematoxylin. 1160 X.

Fig. 20. Stages of sporulation. Iron-hematoxylin. 1860 X.

Fig. 21. Cells with "polar caps" of nuclear material. Not a typical chromatin arrangement. Iron-hematoxylin. 1860 X.

Fig. 22. Stages in sporulation. One cell in oval stage, lightly stained; one showing two nuclei, joined together; one nearly matured spore. Gentian violet-iodine. 1860 X.

Fig. 23. Cells with one, two and four chromosomal bodies. Gentian violet-iodine. 1860 X.

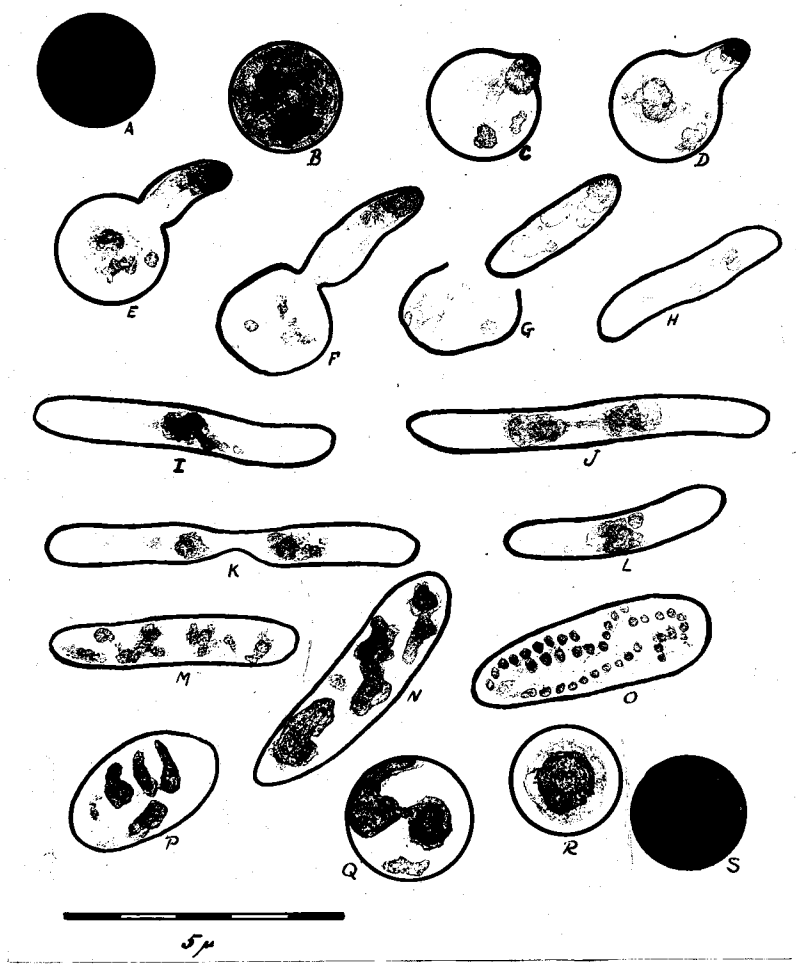
Fig. 24. Late stage in spore formation. Iron-hematoxylin. 1860 X.

Fig. 25. Stages in spore formation. One cell containing four deeply stained bodies. Iron-hematoxylin. 1160 X.

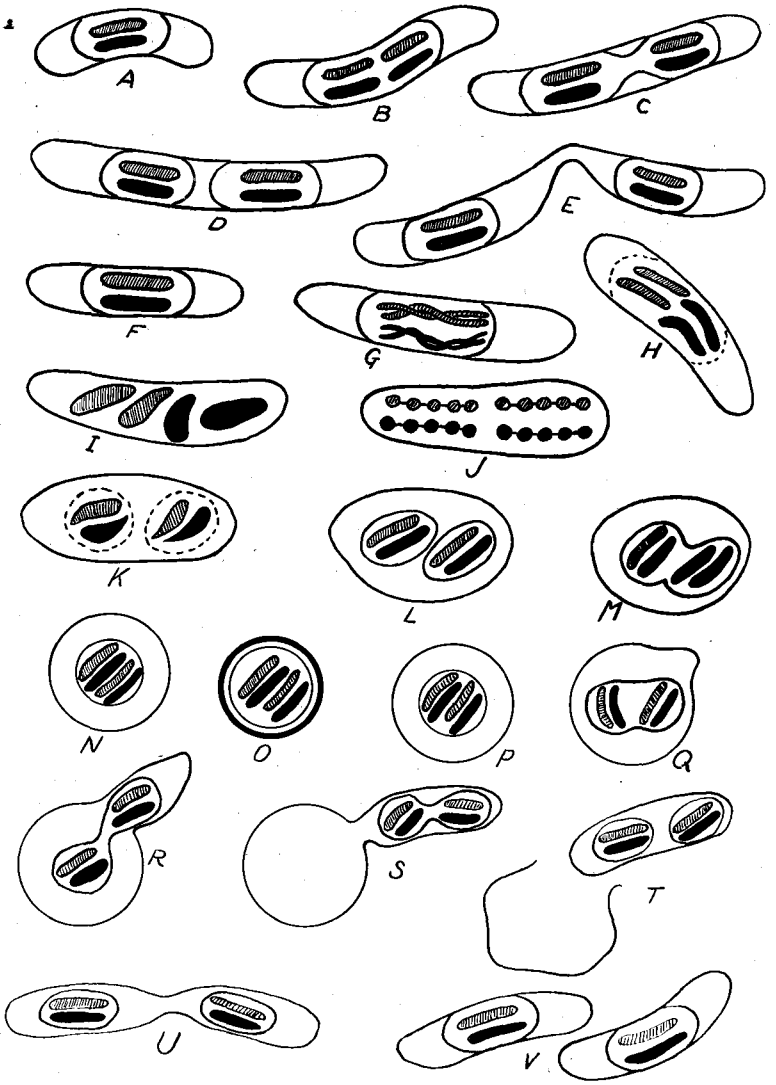
Fig. 26. Various stages in sporulation. Iron-hematoxylin. 1160 X.

Fig. 27. Same. Gentian violet-iodine. 1860 X.

Fig. 28. Migration of cells toward fruiting center. The lightly stained material appearing in "folds" is the slime upon which the cells rest. (Wrinkled during preparation.) Matured spores on left; cells in vegetative and transitional stages at center. Note binucleate cell above left of center. Iron-hematoxylin. 1160 X.



Sec. III, Fig. 1



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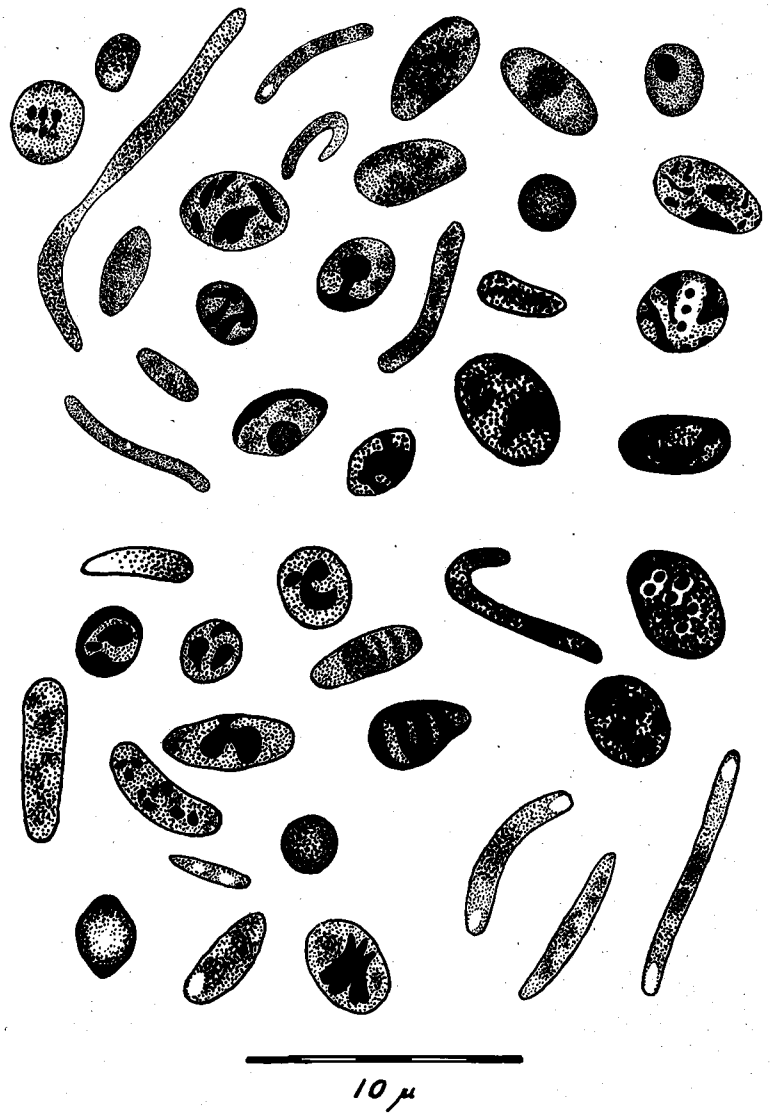

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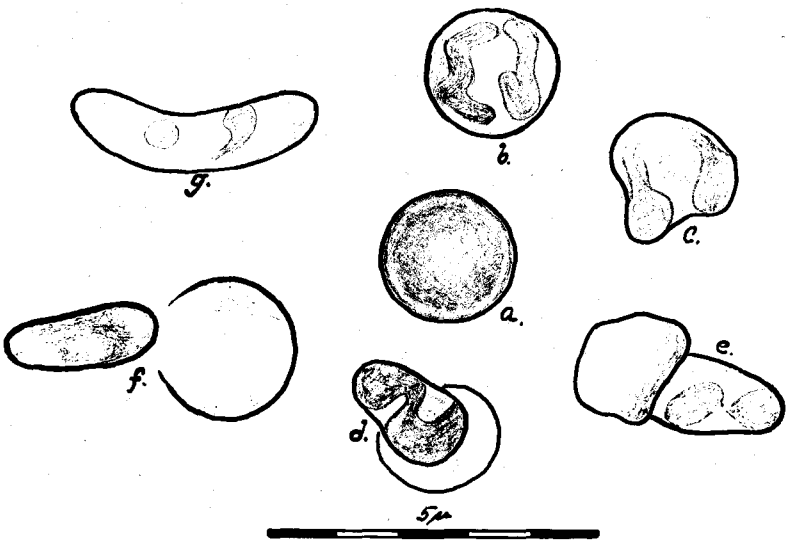
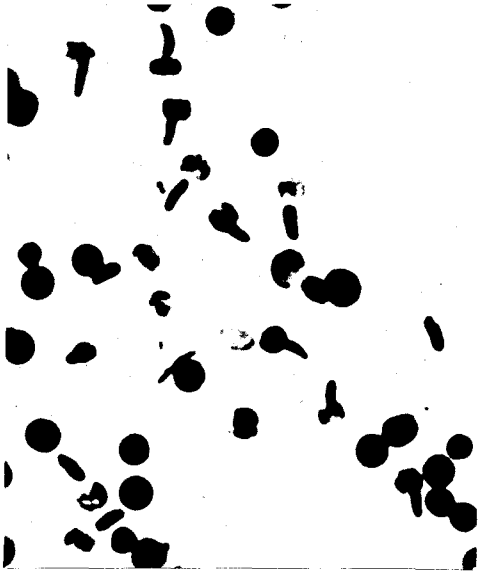


Sec. III, Fig. 4

Sec. III, Fig. 5



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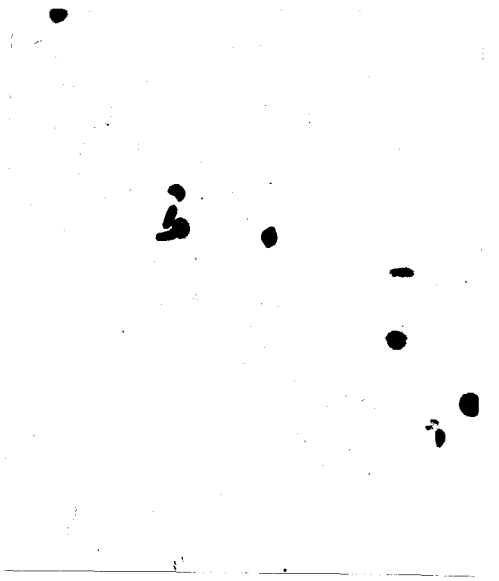
Sec. III, Fig. 7



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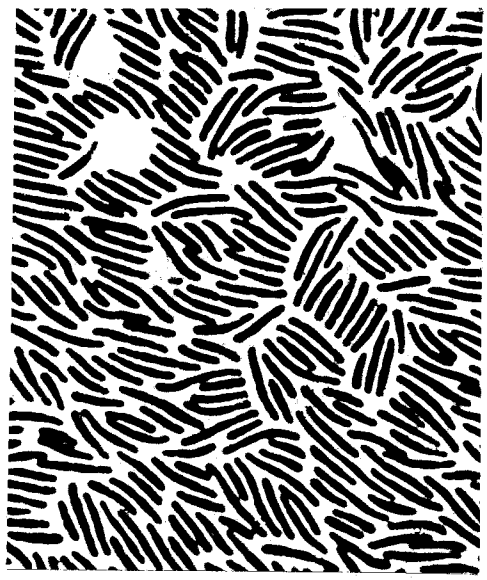

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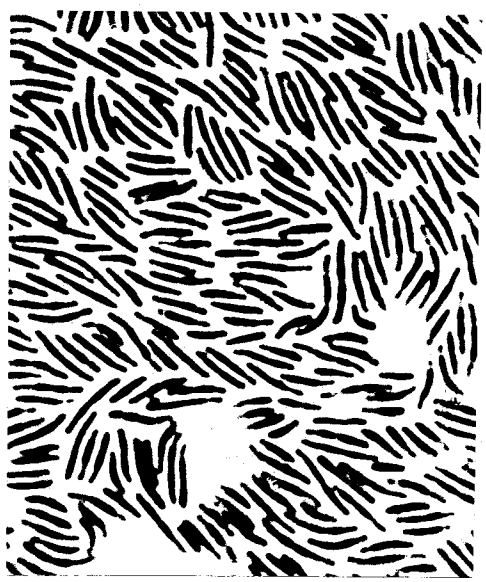

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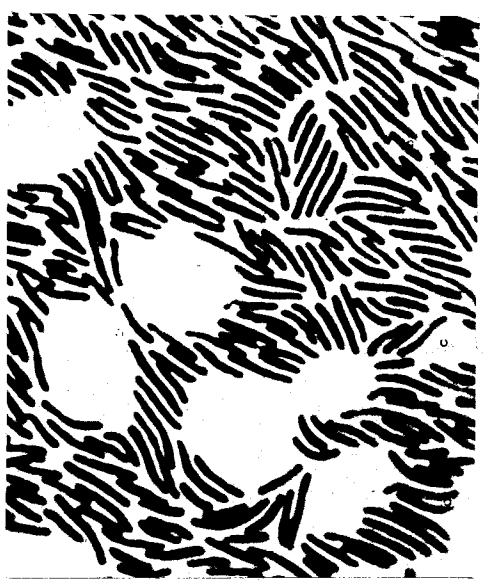

Sec. III, Fig. 11

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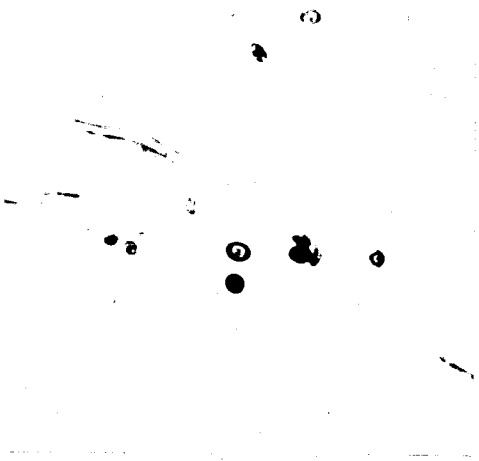
Sec. III, Fig. 23

Sec. III, Fig. 24



Sec. III, Fig. 25

Sec. III, Fig. 26

Sec. III, Fig. 27

Sec. III, Fig. 28

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